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13. ABSTRACT (Maximum 200 Words)
This is the second year of a four-year project to evaluate neurotoxicity related to depleted uranium (DU) exposure and stress in rats. In this year, the project was modified based upon a scientific review of our project at the Force Health Protection Review of Depleted Uranium Projects. This resulted in addition of a pilot stress study, modification of the acute toxicity study and use of an enhanced stress model for the latter and the forthcoming long-term toxicity study. Studies performed in the year being reported included the pilot stress study, which compared several models of stress administered over 6-week period. Based upon serial plasma corticosterone evaluations over the course of the study, it was determined that a weekly regime of four days restraint followed by one of swimming elicited periodic (swimming-related) sharp peaks of corticosterone. This approach was adopted to provide stress for the DU/stress interactions that form the rest of the project. The acute study will be carried out in five blocks, using soluble DU exposures of 0.1, 0.3, and 1.0 mg/kg im, along with the indicated stress. This study employs detailed clinical neurobehavioral evaluation. At sacrifice (on days 1, 3, 7, and 30) icp-ms determination of regional brain uranium kinetics, and neurochemical and neuropathological evaluations will be done. Block 1 of the 5 has been completed.

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Introduction

This is a four-year study on the neurotoxic potential of depleted uranium (DU) in laboratory rats (during the past year the project was converted from a three- to a four-year study, to accommodate an increased scope). Previous studies with Gulf War veterans and experimental animals exposed to embedded DU suggest that neurotoxicity may result from DU exposure (McDiarmid *et al.* 2000; Pellmar *et al.*, 1999). The current investigation is designed to assess the neurotoxic potential of acute and chronic exposure to DU and the contribution of stress to expression of DU neurotoxicity and kinetics. All studies are being performed with adult male Sprague-Dawley rats. Dose-finding and preliminary toxicokinetic studies of DU were conducted in the first year of the project, and reported at the Force Health Protection Review of Depleted Uranium Projects, December 2002. Based upon recommendations from that review, a pilot comparative stress study was conducted, to obtain an optimal model of that condition for use in the acute and chronic DU exposure studies.

Evaluations of neurotoxicity related to acute exposure to uranyl acetate (soluble DU) are in progress, while forthcoming chronic exposures will utilize implanted solid DU particles. Methods being employed include then following. Inductively coupled plasma-mass spectrometry (ICP-MS) analysis will be used to assess the kinetics of uranium in the cerebral cortex, hippocampus, striatum, and cerebellum between 24 hours and 30 days after a single DU administration (in the acute study). Neurotoxicity is assessed with behavioral, morphological, and biochemical endpoints. Behavioral assessment of neurotoxicity utilizes the Functional Observation Battery (FOB), motor activity, and tests of learning and memory (by passive avoidance). Biochemical analyses include quantification of neurotransmitters (dopamine, norepinephrine, serotonin, glutamate, GABA), determination of receptor number and indicators of oxidative stress (levels of oxidized and reduced glutathione), done in the same brain regions examined for DU kinetics. Morphological studies employ perfusion-fixation, multilevel sampling of the nervous system and contemporary light microscopic procedures to allow detailed evaluation of any lesions. Based upon the above noted pilot stress study, a model of daily restraint with intermittent swimming stress is employed, in association with DU exposure These studies will help define the neurotoxic potential of DU and assess the role of stress in modifying this effect.

This is the annual report for year two of this four year study, the major activities of which involved presentation and review of data from the dose-finding and preliminary toxicokinetic studies of year one by an expert panel at the Force Health Protection Review, Performance of a pilot comparative stress study to assess the most relevant stress model to use with the acute and chronic DU exposures, and the initiation of the acute studies of DU neurotoxicity. The progress of the project is indicated in the following table.

Progress of Award # DAMD17-01-0775, Multifactorial Assessment of Depleted Uranium Neurotoxicity as of October 31, 2003 (end of Year 2)

Task	Status
1. Preliminary studies for dose range finding for acute and chronic studies	complete
2. Toxicokinetic study: a. Determination of serum and regional brain uranium concentrations b. Determine the effects of stress on DU kinetics	complete
3. Pilot stress study	complete
4. Acute toxicity study	incomplete (1/5 experimental blocks completed); will be completed in Year 3, assays in progress
5. Chronic toxicity study	to be initiated 2nd half of Year 3

Body

I. Tasks from Statement of Work (as revised 1/10/03) and Accomplishments- This is a complex, four-year study on the neurotoxic potential of depleted uranium and its modulation by stress. What follows are tasks described in the approved Statement of Work (see Appendix), along with relevant research accomplishments for Year 2.

a. ***Task 1***- Perform **preliminary studies** to identify appropriate doses of DU for kinetic studies.

Research Accomplishments for Task 1. The experimental work was performed in Year One, and reported in the October 2002 Annual Report. During the current year, this data was presented for at the Force Health Protection Review of Depleted Uranium Projects in December 2002 (see below). After reviewing our findings, the panel recommended single doses of 0.1, 0.3 and 1.0 mg/kg be employed in the acute study, since these seemed the best scheme to allow measures of neurotoxicity without the confounding issue of renal toxicity.

b. ***Task 2***- Perform a **toxicokinetic study**. This is to determine the concentration of DU in rat serum, brain cortex, hippocampus, striatum, and cerebellum at several times after DU exposure. The effect of stress on DU kinetics will also be determined.

Research Accomplishments for Task 2. This portion of the project was conducted in Year One, and was reported in the October, 2002

Annual Report and the Force Health Protection Review. This is to be submitted for publication in the Journal of Toxicology and Environmental Health, with the manuscript in progress (Barber *et al.*). This will be submitted as an addendum to this report concurrent with its submission to the journal.

c. ***Task 3***- Perform a **pilot stress study**, <u>to determine the most appropriate model of stress to employ in subsequent acute and long-term DU neurotoxicity studies.</u> This was added to the revised Statement of Work following review of our project at the Force Health Protection Review of Depleted Uranium Projects in December 2002. The objective was to determine an acute stress model for obtaining a peak plasma corticosterone level at the time of DU exposure. The hypothesis, based upon previous work in our laboratory investigating various stress models, was that rats stressed by restraint for 4 days and then introduced to the novel stress of swimming would produce a peak corticosterone level comparable to that seen in a previous experiment of 28 days restraint stress followed by one exposure to novel swim stress. A second objective of the pilot study was to investigate the stress model to be used in the chronic DU neurotoxicity study by determining whether similar spikes in plasma corticosterone levels will occur over a prolonged exposure to the 4 day restraint/1 day swim model. This was accomplished by comparing several stress models in Sprague-Dawley rats using serial measures of body weight and plasma corticosterone concentration (blood samples were collected from the orbital sinus within 10 minutes after cessation of the stress episode). The stress models were (all administered 5 days/week for 6 weeks, n=8/group): Group 1- handling (control); Group 2- restraint for 30 minutes; Group 3- 4 days restraint for 30 minutes, followed by 1 day swimming for 30 minutes; Group 4- swimming for 30 minutes. The schedule of the study was as follows (Table 1).

Table 1. Schedule of activities for the pilot study.

SUN	MON	TUES	WED	THURS	FRI	SAT
		Day-6	Day -5	Day -4	Day -3	Day -2
		Acclimation to blood collection procedures			• BW* • blood *	
Day -1	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
	• stress	• stress	• stress	• stress	• stress • BW • blood	
Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12
	• stress	• stress	• stress	• stress	• stress • BW • blood	
Day 13	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19
	• stress	• stress	• stress	• stress	• stress • BW • blood	
Day 20	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26
	• stress	• stress	• stress	• stress	• stress • BW	
Day 27	Day 28	Day 29	Day 30	Day 31	Day 32	Day 33
	• stress	• stress	• stress	• stress	• stress • BW • blood	
Day 34	Day 35	Day 36	Day 37	Day 38	Day 39	Day 40
	• stress	• stress	• stress	• stress	• stress • BW	
Day 41	Day 42	Day 43				
	• stress • BW	• sacrifice • blood				

* BW= body weight blood= blood collection for corticosterone determination

Results of the study supported the hypothesis that corticosterone levels are elevated in animals stressed by restraint for 4 days (Mon-Thurs) and swimming on day 5 (Friday) (Figure 1).

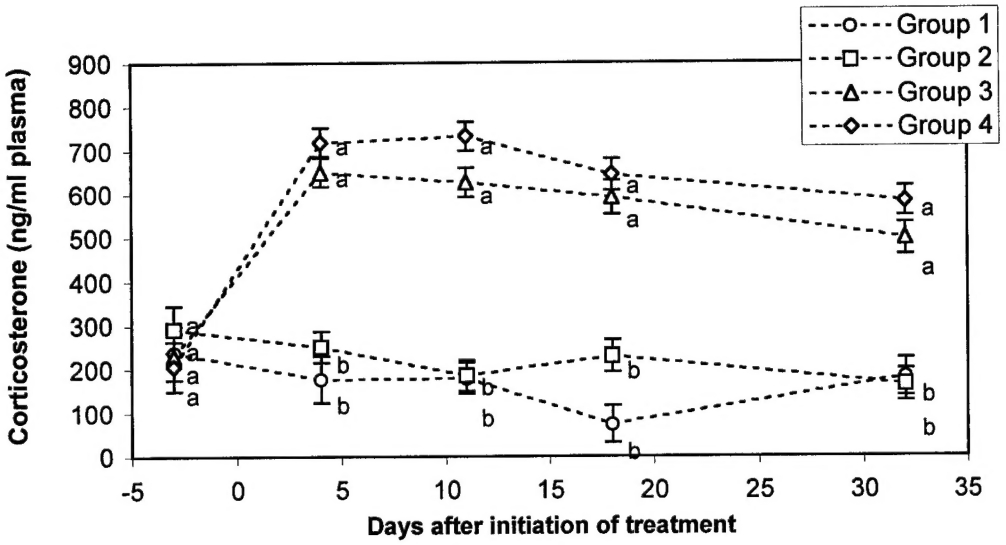

Figure 1. Corticosterone levels of animals by treatment group and study day. Blood for plasma corticosterone analysis was collected within 10 minutes after the cessation of the fifth day of weekly stress (Table 1). Each point represents the mean of 8 observations (± standard error of the mean). Means within a day with no letters in common are significantly different at α=0.05 according to Bonfferoni-corrected multiple comparison.

Plasma corticosterone levels in Groups 3 (restraint/swim) and 4 (swim) were significantly elevated ($p<0.05$) above Groups 1 (handle) and 2 (restraint) on days 4, 11, 18 and 32, but they were not statistically different from each other. Likewise, Groups 1 and 2 did not differ significantly. The elevated plasma corticosterone peaks on the 5th day of weekly stress continued over the 6-week exposure for Groups 3 and 4. In order to approximate the levels of corticosterone before and after stress on the first 4 days of the week, animals sacrificed on day 43 (Tuesday) were divided into 2 subgroups. In each treatment group, 4/8 animals were stressed prior to sacrifice and the remaining 4/8 animals were not so stressed. The results of the corticosterone assays for these animals are shown in Figure 2.

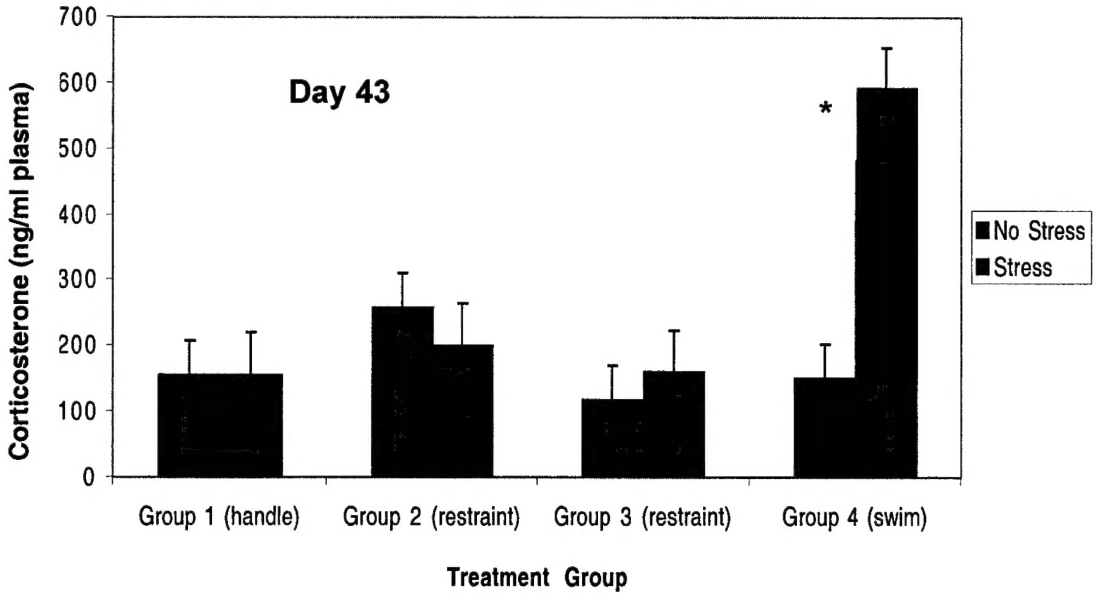

Figure 2. Corticosterone levels of animals by treatment group on study day 43 (2nd day of weekly stress- see Table 1). Stress/no stress indicates presence or absence of presacrifice stress episodes in each group. Blood for plasma corticosterone analysis was collected within 10 minutes after the cessation of stress. Each point represents the mean of 4 observations (± standard error of the mean). * indicates a significant difference between no stress and stress in that treatment group at α=0.05 according to test of simple main effect.

These findings indicate that elevation of plasma corticosterone seen in Groups 3 and 4 (Figure 1) is directly associated with swimming stress, and that such elevations are transient. As an example, Group 4 rats that were swum on day 43 had elevation of plasma corticosterone (Figure 2, Group 4, stress). Other members of that group, who had last swum on day 42 (the day before blood samples were obtained, Table 1) had normal levels of this hormone on day 43 (Figure 2, Group 4, no stress). It appears that post-swimming elevation of this hormone returned to normal within 24 hours. Similarly, Group 3 rats, last swum on day 39, had normal plasma corticosterone levels on day 43, whether or not preterminal restraint was applied (Figure 2).

Note: An ancillary investigation was conducted at the end of the study with these animals, to see if these stress models would affect acute (cholinergic) toxicity of the organophosphate chlorpyrifos. The latter was administered on day 39 (after data for Figures 1 and 3 were collected) to 4/8 animals/group. Sacrifice was on day 43. From the Hancock et al. abstract (Appendix), there was no stress-related alteration of brain chlorpyrifos-induced acetylcholinesterase inhibition. In addition, there was no chlorpyrifos associated alteration of plasma corticosterone. Thus, the data displayed in Figure 2 represents a combination of chlorpyrifos dosed and not dosed rats to give the simple main effect of presacrifice stress. This approach permitted us to obtain additional data and still meet the requirements of the Statement of Work for the pilot stress study.

Stress also affected body weight beginning on day 18 (Figure 3). Body weights of animals in Groups 2, 3 and 4 were consistently lower than control Group 1, but only Group 4 animals were significantly different ($p<0.05$) from Group 1.

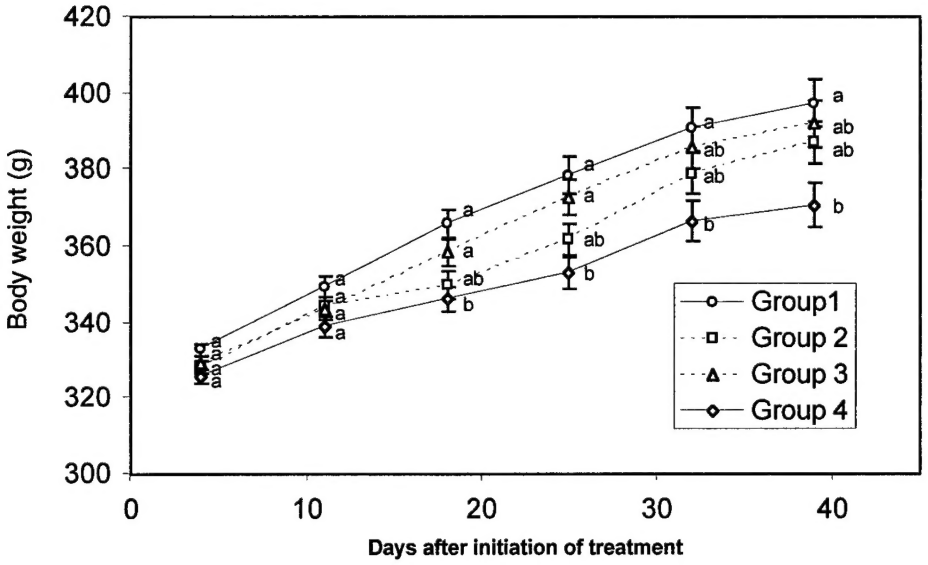

Figure 3. Body weight by treatment group and study day. Each point represents the mean of 8 observations (± standard error of the mean). Means within a day with no letters in common are significantly different at $\alpha=0.05$ according to Bonfferoni-corrected multiple comparison.

Data from this study are to be presented at the November 2003 meeting of the Society for Neuroscience (see Hancock *et al.* abstract in the Appendix). In addition, the cerebral hemispheres from four rats of each group have been sent to Dr. Ronald Mervis, NeuroStructural Research Laboratories, Tampa, FL. He will do Golgi staining and assess the conformation and size of hippocampal neurons (dentate gyrus, CA1 and CA3). In addition, Dr. Mervis will perform quantitative analysis of synaptic spines on CA3 pyramidal cells. This has proven to be a sensitive measure of synaptic plasticity in neuronal injury (Morest, 1981, Flood *et al.*, 1993). Extra regional brain samples from all rats in the study are also being frozen at - 70°, and will be sent to Dr. Michael Aschner, Wake Forest University Medical Center. He will perform assays of divalent metal transporter-1 levels, a measure of potential uranium transport from blood to brain.

d. ***Task 4***- Perform an **acute toxicity study** evaluating the effects of several doses of soluble DU and of stress, as measured by neurobehavioral, neurochemical and neuropathological and regional brain uranium concentrations. This was modified from the original Statement of Work, based upon review of the initial year's results at the December 2002 Force Health Protection Review. The current plan is to use single intramuscular doses of 0.1, 0.3 and 1.0 mg/kg of uranyl acetate in male Sprague-Dawley rats, as recommended by the Review Panel. The highest of these doses is the lowest used in our kinetic studies, but these avoid the issue of severe uranium nephrotoxicity seen in the preliminary studies. The current acute toxicity study includes the following,

(1) One week of administration of stress consisting of four consecutive days of restraint stress followed by one day of forced swimming. At the cessation of stress, animals are bled for subsequent corticosterone determination and immediately dosed intramuscularly with DU. This resulted in transient elevation of plasma corticosterone at the time of dosing (see below).
(2) Weekly neurobehavioral in-life assessments (Functional Observational Battery, motor activity, passive-avoidance.
(3) At sacrifice (days 1, 3, 7 and 30), neurochemical evaluation (n= 5/group) of regional brain neurotransmitters, their receptor levels and the intensity of oxidative stress (see Appendix, Methods, p. 24 for list of neurochemical assays); ICP-MS analysis of blood and regional brain uranium levels (n= 5/group); detailed neuropathology (n=5/group, not done on sacrifice day 1); and clinical biochemistry (n=5/group). The overall n/group is thus up to 15, and total number in the study is 418 rats. Thus, the current design has four treatments (three dose levels of DU plus control), four sacrifice intervals (days 1, 3, 7, 30), and two stress states (yes or no). This number of rats and workload (such as daily stress) necessitates performing the in-life portion of the study in five periods (blocks). The experimental design for each block follows.

Table 2. Treatment design for each experimental block of the acute study.

Group	Stress	DU dose	Total Animals	n/group	Day 1	Day 3	Day 7	Day 30
1	no	0	pathology*=3, neurochem*=4, icp-ms*=4	11	pathology =0 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1
2		0.1	pathology=3, neurochem=4, icp-ms=1	8	pathology =0 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=0	pathology =1 neurochem=1 icp-ms=0	pathology =1 neurochem=1 icp-ms=0
3		0.3	pathology=3, neurochem=4, icp-ms=4	11	pathology =0 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1
4		1	pathology=3, neurochem=4, icp-ms=4	11	pathology =0 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1
5	yes	0	pathology=3, neurochem=4, icp-ms=4	11	pathology =0 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1
6		0.1	pathology=3, neurochem=4, icp-ms=1	8	pathology =0 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=0	pathology =1 neurochem=1 icp-ms=0	pathology =1 neurochem=1 icp-ms=0
7		0.3	pathology=3, neurochem=4, icp-ms=4	11	pathology =0 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1
8		1	pathology=3, neurochem=4, icp-ms=4	11	pathology =0 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1	pathology =1 neurochem=1 icp-ms=1

*pathology = animals used for neuropathological and kidney lesion evaluation
neurochem = animals used for neurochemical evaluation of regional brain transmitters, receptor levels and oxidative stress
icp-ms = animals used for inductively coupled plasma-mass spectrometry analysis of uranium concentration

Below is the study schedule for each experimental block:

Table 3. Schedule of activities for each experimental block of the acute study.

SUN	MON	TUES	WED	THURS	FRI	SAT
Day -12	**Day -11**	**Day -10**	**Day -9**	**Day -8**	**Day -7**	**Day -6**
		animals out of quarantine			rotarod training	
Day -5	**Day -4**	**Day -3**	**Day -2**	**Day -1**	**Day 0**	**Day 1**
	rotarod training passive avoidance (PA) training	rotarod training PA retention/retrain		Functional Observational Battery (FOB) Motor Activity (MA)	restraint stress anesthesia chamber adaptation	restraint stress anesthesia chamber adaptation
Day 2	**Day 3**	**Day 4 (Dosing day)**	**Day 5**	**Day 6**	**Day 7**	**Day 8**
restraint stress anesthesia chamber adaptation	restraint stress anesthesia chamber adaptation	swim stress (a.m.) blood collection (for CORT assay) and **DU injection after stress**	**Sacrifice** -24 hour post-dosing		**Sacrifice** 3 days post-dosing	
Day 9	**Day 10**	**Day 11**	**Day 12**	**Day 13**	**Day 14**	**Day 15**
	FOB/MA	**Sacrifice** 7 days post-dosing	PA retention			
Day 16	**Day 17**	**Day 18**	**Day 19**	**Day 20**	**Day 21**	**Day 22**
	FOB/MA		PA retention			
Day 23	**Day 24**	**Day 25**	**Day 26**	**Day 27**	**Day 28**	**Day 29**
	FOB/MA		PA retention			
Day 30	**Day 31**	**Day 32**	**Day 33**	**Day 34**		
	FOB/MA		PA retention	**Sacrifice** 30 Day post-dosing		

The first of these blocks (designated experiment DUA-1) was completed in October 2003, with the next four being scheduled for the January- May 2004 period. For the DUA-1 experiment, the average plasma corticosterone levels of unstressed (Groups 1-4) and stressed (Groups 5-8) animals at the time of DU dosing were 170.7 ng/ml (Standard Deviation [SD] = 74.4, range = 37.4 - 355.1) and 667.1 ng/ml (SD=92, range = 466.1 - 856.1), respectively.

Since this is only 1/5 of the acute study, data is incomplete. For neurobehavioral observations, only animals designated for the 30-day post-dosing sacrifice were used. As a result there is a current n=2-3 for each treatment group at each data collection day. Once all experimental blocks are completed, the data will be pooled giving an n=10-15/treatment group at each day. Due to the current small sample size it is difficult to ascertain trends in the behavioral data at this time.

The samples from DUA-1 for pathological study are divided into two endpoints: tissues for paraffin embedding, which have been embedded and are awaiting sectioning, and tissues for epoxy resin embedding, which have been processed and embedded and are currently being sectioned. Samples for neurochemical analyses have been prepared and are currently being analyzed. Samples for ICP-MS analysis are being prepared for analysis, and will be shipped to the Analytical Section of the Hazard Identification Core in the Southwest Hazardous Waste Program, University of Arizona for that purpose.

Blood samples collected for clinical biochemistry have been analyzed for experiment DUA-1, with 2 rats/dose at each sacrifice interval. These data indicate that mid- and high-doses (0.3 or 1.0 mg/kg DU) led to modest elevation of serum blood urea nitrogen (BUN) on post-dosing days 3 and 7. These were in the range of 40 to 60 mg/dl (control levels were 23-29 mg/dl), being greater with the higher dose and on day 3. In addition, there was concurrent elevation of serum creatinine and some body weight loss (possibly due to dehydration) in these rats. The creatinine levels were 1.9 and 2.1 mg/dl on day 3 and 1.2 and 1.4 on day 7 for the 1.0 mg/kg DU rats (n=2). For the 0.3 mg/kg rats, these values were 1.3 and 1.3 on day 3 and 0.7 and 0.7 on day 7. Control animals values were 0.4 mg/dl. There was return of BUN and creatinine levels to normal on day 30. Stress did not affect these values.

e. ***Activities outside Statement of Work***- We presented results from studies conducted during the first year (October 2001-September 2002) at the Force Health Protection Review of Depleted Uranium Projects, December 2002, Albuquerque, NM. The review panel consisted of Drs. Robert Ullrich, Deborah C. Rice, Naomi H. Harley, Marie-Helene Henge-Napoli, William L. Daniels, and Sharleas Hunter. Copies of the slides containing the presented data are included in the Appendix. The major aspects of the presentation were as follows. There was an overview of the study design and methods, along with the major requirements of the Statement of Work. Data from the preliminary study to establish doses of soluble DU (as uranyl acetate) to be used in subsequent acute neurotoxicology experiments, and to determine the kinetics of uranium following such exposure were presented (detailed description of this work was provided in the October 2002 Annual Report). The single doses (10, 30 and 100

mg/kg) were toxic, but did demonstrate dose-dependent elevations of uranium in serum, kidney and brain at 24 hours post-dosing. The subsequent stress/kinetic study of soluble DU used single doses of 10 or 1 mg/kg (see October 2002 Annual Report for details), and revealed ICP-MS detected increased uranium concentrations in regions such as the cortex, hippocampus, caudate-putamen (striatum) and cerebellum, often in a dose-related fashion in the 8 hour-10 day post-dosing period. By 30 days concentrations were within control limits in the 1 mg/kg group (the 10 mg/kg animals had to be sacrificed at 10 days due to renal toxicity). Stress, in the form of 5 daily forced swimming periods prior to the DU exposure had no significant effect. Additional data reported to the Review Panel related to the pathological and heat shock protein changes induced by these doses of DU in kidneys, and of a preliminary comparative stress study. The Review Panel suggested several changes in the project to improve its scientific and military relevance. We accepted these recommendations, and these are and incorporated into the revised (1/10/03) Statement of Work which currently guides the project. These were:

1- Development of a pilot study to refine a better stress model for use in the acute and chronic toxicity studies is to be done.

2- Changes in the acute (with soluble DU) and chronic (with implanted pelleted DU) toxicity studies, as follows,

a- Acute toxicity- relative to DU administration this will have an antecedent enhanced stress model (measured by plasma corticosterone levels), and will include additional rats so that brain and blood uranium kinetics can be performed using inductively coupled plasma-mass spectroscopy (ICP-MS) assays. In addition, the uranium exposure will be changed, using lower doses and a third dosage group (control plus 1.0, 0.3 and 0.1 mg/kg DU). Another sacrifice interval (day 3) will be added.

b. Long-term (chronic) toxicity- The prolonged exposure to implanted DU will be concurrent with long-term modified stress. The latter is designed to provide periodic peaks of elevated plasma corticosterone during the six-month study. Five rats per group are added to provide for ICP-MS study of blood and regional brain uranium concentration, determined at sacrifice.

3- The gene expression study using microarrays as described in the original Statement of Work, is deleted.

III. Problems Encountered, Solutions, and Recommended Approaches to Future Work.

a. Acute toxicity study schedule. As noted above, this is to be conducted in five blocks, to accommodate the numbers of rats in the study and the complex in-life stress and clinical evaluations procedures that are needed. The planned schedule has the in-life portion of this acute

study completed by the end of month 26 (December, 2003. We have completed the in-life portion of the first block of the study, and plan to complete the remaining ones by the end of month 32 (May 2004). Reasons for this delay included the need to revise the study to address issues raised by the Review Panel at the Force Health Protection Review of Depleted Uranium Projects. Our contention is that this enhances the scientific value of the project, but did require time for changes in planning and approach and for the requested pilot stress study. There is sufficient flexibility built into the schedule of the Statement of Work to allow for this delay and still complete the entire project on time.

b. Toxicity of 1 mg/kg dose of uranyl acetate- Mild to moderate transient nephrotoxicity was noted with the mid- and high- dose (0.3 and 1.0 mg/kg) of soluble DU (see above). This will need to be factored into the evaluation of neurotoxicological data from these animals. Since we have both the latter data and clinical pathological findings from individual animals, these correlations can be made on an individual as well as on a group basis.

Key Research Accomplishments- Year Two

- Presentation of year one data at the Force Health Protection Review of Depleted Uranium Projects, and revision of the study to address concerns of the Review Panel.

- Completion of the Pilot Stress Study and selection of a stress model (daily restraint/periodic forced swimming) to employ during the remainder of the project. Acceptance of a paper on this data for presentation at the 2003 Society for Neuroscience meeting (see Hancock *et al.* in the Appendix)

- Initiation of the acute toxicity study with soluble depleted uranium.

- Presentation of three papers at the 2002 meeting of the Society of Toxicology (references cited in Toxicol. Sci. 72 (supplement), 2003: Barber and Kopplin' Regional distribution of uranium in rat brain, p. 19; Munson *et al.* Heat shock protein and uranium nephrotoxicity, p. 347; Pomeroy *et al.* Uranium and cell death in the rat kidney, p. 16).

- Acceptance of one paper for presentation at the November, 2003 meeting of the Society for Neuroscience (see Hancock *et al.* abstract in Appendix).

- Two papers in preparation:

  Barber, D.S., Ehrich, M. Jortner, B.S. Distribution and kinetics of uranium in the rat brain after intraperitoneal injection of uranyl acetate, for submission to Journal of Toxicology and Environmental Health.

  Tolson K, Pomeroy M, Roberts S, Barber D. Role of heat shock proteins in resistance to uranium nephrotoxicity, for submission to Journal of Toxicology and Environmental Health.

  Copies of these will be sent as an addendum to this report concurrent with their submission to the journal.

Reportable Outcomes

One abstract was accepted for the November 2003 annual meeting of the Society for Neuroscience-

S.K. Hancock, M.F. Ehrich, J. Hinckley, T. Pung, K.L. Farris, B.S. Jortner. Stress and neurotoxicant exposure. Comparison of effects of several stress models on the acute neurotoxicity of the organophosphate chlorpyrifos (see Appendix).

Presentation of three papers at the 2003 meeting of the Society of Toxicology

REGIONAL DISTRIBUTION OF URANIUM IN RAT BRAIN. D S Barber[1] and M J Kopplin[2]. [1] Center for Environmental and Human Toxicology. University of Florida, Gainesville, FL, USA; [2]Department of Pharmacology and Toxicology, University of Arizona, Tucson, AZ, USA. Toxicol. Sci. 72 (supplement):19, 2003.

HEAT SHOCK PROTEINS AND URANIUM NEPHROTOXICITY. J W Munson[1], J K Tolson[1], B S Jortner[2], S M Roberts[1] and D S Barber[1] . [1]Center for Environmental and Human Toxicology. University of Florida, Gainesville, FL, USA; [2]Laboratory for Neurotoxicity Studies, VPI&SU, Blacksburg, VA, USA. . Toxicol. Sci. 72 (supplement):347, 2003.

URANIUM AND CELL DEATH IN THE RAT KIDNEY. M Pomeroy[1],B Jortner[1],M Ehrich[1],J Robertson[1], and D S Barber[2]. [1]Department of Biological Sciences and Pathobiology, Virginia Tech, Blacksburg, VA, USA; [2]Department of Physiological Sciences, Center for Environmental and Human Toxicology, University of Florida, Gainesville, FL, USA. Toxicol. Sci. 72 (supplement):16, 2003.

Conclusions

The project was revised to meet the recommendations of the Review Panel of the Force Health Protection Review of Depleted Uranium Projects (December 2002). The major changes included development of a better stress model, reduction of depleted uranium doses in the acute stress study (to reduce nephrotoxicity), more extensive study of the kinetics of uranium in brain regions during the acute and long-term studies.

A six-week long pilot stress study revealed there was elevation of plasma corticosterone and reduction in body weight across the study. These were noted in rats receiving daily swim stress and in those having restraint stress for four consecutive days followed by one day with swimming. The plasma corticosterone elevations were related to the swimming episodes. No significant elevations were noted in rats exposed to routine handling (control group) or daily restraint stress.

The acute toxicity study using soluble depleted uranium (administered as uranyl acetate) has been initiated, with 1/5 blocks being completed. Definitive conclusions cannot be drawn from this study, since it is incomplete.

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Appendix-

Methods

1. Stress procedures- Stress was induced by a combination of four consecutive days of restraint stress followed by swim stress on the fifth day. For restraint stress, each animal was placed in an individual Plexi-glas® tube (6 cm diameter X 22 cm long) (Konarska, Stewart and McCarty, 1989) with adequate breathing holes for a 30-minute period and then returned to its home cage. For swim stress, each animal was placed in a 4-chambered tank of water at 23°C - 25°C and allowed to swim for a 30-minute period. When swimming was completed, the animal was towel-dried for 1-2 minutes, placed under a heat lamp for an additional 2-3 minutes and returned to its home cage. Animals in the control (no stress) groups were handled daily by removing the animal from its home cage, placing it in a box and immediately returning it to its home cage.

2. Uranium analysis- Uranium analysis was performed by inductively coupled plasma-mass spectrometry (ICP-MS) on samples that had undergone nitric acid/peroxide digestion. Samples were placed in 15ml sealed glass pressure tubes with 0.5 ml of concentrated metal-free nitric acid (Optima, Fisher Scientific). Samples were heated to 140°C for 2 hours in a silicone oil bath, then 0.5ml of 30% hydrogen peroxide (Ultrex II, J.T. Baker) was added and samples heated at 110°C for a further 60 minutes. Samples were cooled, quantitatively transferred to acid-washed 5ml volumetric flasks, and brought to volume with deionized water (ElgaStat Maxima). Uranium analysis was performed by inductively coupled plasma-mass spectrometry (ICP-MS) by the Analytical Section of the Hazard Identification Core in the Southwest Hazardous Waste Program. Analysis was conducted on an HP 7500a ICP-MS using iridium as an internal standard. Uranium concentration was determined from a standard curve of uranium based on the m/z 238 signal. Five repetitions were performed per sample and the average used to calculate uranium concentration. The limit of quantitation of this method was 0.002ppb. Recovery was determined from samples spiked with 0.1-10ppb uranium and determined to be 96-108%. The limit of quantitation of this method under normal conditions was 0.01 ppb. By using a lower range of standards, the limit of quantitation was improved to 0.002ppb to accurately determined uranium concentrations from samples with low levels of uranium. Recovery was determined from samples spiked with 0.1-10ppb uranium and determined to be 96-108%.

3. Corticosterone determination- Blood was collected from the orbital sinus (under isofluorane anesthesia) immediately following the stress session. Whole blood was collected in heparinized microcentrifuge tubes, centrifuged @12000 rpm at 4° C for 4 minutes. The plasma was removed and frozen at -70° C until analysis by a corticosterone ^{125}I-radioimmunoassay kit (ICN Biomedicals, Costa Mesa, CA).

4. Neurobehavioral Evaluations-
   The Functional Observational Battery (FOB) provides a range of clinical/behavioral evaluations, which include measures of behavioral and CNS excitability (home cage posture, activity rearing, arousal, ease of handling and removal from cage, involuntary movements, stereotypy, bizarre behavior); autonomic effects (salivation, lacrimation, urination, defecation, piloerection); effects on muscle tone and equilibrium (gait, fore-and hindlimb grip strengths, foot splay, duration/agility on rotarod); effects on motor and sensory systems (response to approach, touch, sound and tail pinch); and effects on the general physiology (weight, body temperature, respiration) (Moser *et al.*).

   The horizontal motor activity level of animals is measured using an automated cage rack photobeam activity system. Motor activity is measured by counting interruptions of photobeams in a cage at 2-minute intervals over a period of 30 minutes. This system provides measurements of 1) non-ambulatory or fine movements by counting repeated breaks of the same photobeam, 2) ambulatory or larger locomotor movements by counting breaks of adjacent photobeams and 3) total motor activity by adding counts of all photobeam breaks.

   Lastly, the passive-avoidance test is one that provides measures of learning and memory. Briefly, this involves a single acquisition trial session (training) on Day 0, prior to corticosterone dosing. The training consists of a 30-second adaptation in a darkened chamber prior to the trial onset. The trial begins with the onset of a bright light in the chamber containing the rat. To avoid the light, the rat can cross over into a darkened chamber at which time the animal is given a slight footshock for 3 seconds. The measurement recorded (latency) for each animal is the length of time taken to cross over to the darkened chamber. The maximum trial length is 180 seconds. The training trial is followed by a retention trial, which mimics training except no footshock is given. Once again, the recorded measurement is the length of time taken to cross over to the darkened chamber. The retention trials are performed at various intervals throughout the study period. Due to the placement of the training and retention trials in the study schedule, the passive avoidance measurements are largely a test of memory.

5. Pathology- Following perfusion-fixation with 4% paraformaldehyde and 0,2% glutaraldehyde, cross-sections of medulla, and spinal cord (cervical, thoracic and lumbar), peripheral nerve (sciatic, tibial, sural, vagus), optic nerve, and longitudinal sections of dorsal root ganglion and associated spinal nerve roots were embedded in Polybed epoxy resin, sectioned at 1 μm thickness and stained with toluidine blue and safranin for light microscopic study. Cross-sections of the frontal, parietal, and occipital

levels of the cerebral hemispheres, midbrain, cerebellum and pons, and kidney and longitudinal section of the ocular globe (rostro-caudal plane) were embedded in a mixture of paraffin, sectioned at 5 µm thickness and stained with hematoxylin and eosin, Fluoro-Jade (a fluorescent stain to detect degenerating neurons- Schmeud and Hopkins), and immunostained for glial fibrillary acidic acid.

6. Neurochemistry- Half of the brain tissue (cerebellum, cortex, hippocampus and caudate putamen) from each animal was homogenized in 0.1M perchloric acid, centrifuged and the supernatant aliquoted into three tubes for the following analysis endpoints:
    a. HPLC determination of catecholamines (dopamine, serotonin and norepinephrine) (methods of Jussofie *et al.*, 1993).
    b. Glutathione (oxidized and reduced) determination
    c. Glutamate and GABA determinations (methods Yasumata *et al.*, 1998).

    The remaining half of the tissue regions was frozen at -70 C for subsequent determination of receptor numbers for regional neurotransmitters (D_1 and D_2 dopamine, serotonin 1a, GABA and acetylcholine receptors), using saturation radioligand binding.

7. Statistical Analysis- Responses measured each day of the pilot stress study were separately subjected to analysis of variance with mean separation by Bonferroni-corrected multiple comparisons. All calculations were performed using the SAS System (version 8.02, SAS Institute Inc. Cary, NC 27513).

Abstract-for presentation at 2003 Meeting of Society for Neuroscience

STRESS AND NEUROTOXICANT EXPOSURE. COMPARISON OF THE EFFECTS OF SEVERAL STRESS MODELS ON THE ACUTE NEUROTOXICITY OF THE ORGANOPHOSPHATE CHLORPYRIFOS. S.K. Hancock, M.F. Ehrich, J. Hinckley, T. Pung, K.L. Farris, B.S. Jortner*. *Laboratory for Neurotoxicity Studies, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA, USA*

Considerable interest exists regarding the possible role of stress in modifying neurotoxicity. We have evaluated several stress models in Sprague-Dawley rats, using serial measures such as body weight, selected neurobehavioral tests and plasma corticosterone concentration (samples were collected within 10 minutes after the stress episode). We then assessed the effect of these stressors on the ability of a neurotoxic organophosphate (the insecticide chlorpyrifos) to inhibit brain acetylcholinesterase. The stress models were (all administered 5 days/week for 6 weeks, n=8/group): Group 1- handling (control); Group 2- restraint for 30 minutes; Group 3- 4 days restraint for 30 minutes, followed by 1 day swimming for 30 minutes; Group 4- swimming for 30 minutes. On day 39 the rats were bled for plasma true acetylcholinesterase (AChE) determination, and then 1/2 the rats in each group (n=4) were administered 60 mg/kg chlorpyrifos subcutaneously. The rats were sacrificed on day 43, and the brains collected for AChE determination. The major stress related findings during the 6-week exposure period included elevated plasma corticosterone. in Groups 3 (restraint/swim) and 4 compared to the controls and Group 2 (restraint) ($p< 0.05$). This was noted from day 4 onward. These levels of corticosterone did not differ significantly between groups 3 and 4. All stress groups had increase in fine motor movements on days 38 and 42. Stress had no effect on plasma AChE levels determined on day 39. Chlorpyrifos administered on that day significantly ($p< 0.05$) inhibited the activity of brain AChE in the control and all 3 stress groups, with no significant differences among them. Thus, none of our stress states modified the intracerebral anticholinesterase effect of 60 mg/kg chlorpyrifos in rats. (Supported by DAMD17-01-1-0775, US Army Medical Research and Materiel Command)

STATEMENT OF WORK- Revised 1/10/03

The proposed work will use the male Sprague Dawley rat to characterize the kinetics and toxicity of depleted uranium (DU) in the brain. The ability of stress to affect disposition and toxicity of DU will also be examined, as stress can alter the permeability of the blood-brain barrier and enhance neurodegeneration. The studies would examine neurotoxicity of DU with and without stress in acute and long-term exposures. Assessment would include detailed morphological, neurobehavioral, neurochemical and toxicokinetic methods.

The experimental design will be a split plot design. The whole plot will be a factorial array in a randomized complete block design. The treatments are stress (2 levels- stressed and unstressed) and DU dose (2-4 levels including negative controls). The whole-plot is rat and the sub-plot is brain region (4 regions). For the **kinetic study**, a 2x2 design will be used (2 doses of DU and 2 levels of stress [stressed or unstressed]). The stressor will be applied once daily for at least 5 days prior to DU exposure. Uranium levels in cortex, hippocampus, caudate-putamen, and cerebellum, as well as in blood, will be determined at several times after DU exposure (e.g., 8 hours, 1 day, 7 days, and 30 days). For the **acute toxicity study**, a 2x4 design will be used, consisting of 2 levels of stress (stressed and unstressed) and 4 levels of DU (control, low, mid and high). For stressed animals there will be daily routine stress with superimposed novel stress just prior to dosing (Hancock *et al.*). At several times after DU exposure (e.g., 1, 3, 7 and 30 days), brain samples will be analyzed for neurotransmitter levels, receptor numbers, evidence of oxidative stress and regional uranium concentrations. At the later time periods (e.g. 3, 7 and 30 days), samples will also be taken for neuropathological study. Neurobehavioral testing will be performed before dosing and weekly thereafter. The acute toxicity study will be performed in a number of experimental blocks. For the **long-term toxicity study**, a 2x4 design will be used with 2 levels of stress (stressed and unstressed) and 4 levels of implanted DU (tantalum negative control, low, medium, and high concentrations of DU pellets). Stress will be induced by frequently (such as 5 days/week) applying a routine stressor with superimposed periodic novel stress throughout the study (using parameters confirmed by a prior **pilot stress study**). Animals will be exposed to DU for 6 months. Behavioral testing will be performed before dosing and every 3 weeks thereafter. After 6 months, samples will be taken for neurochemical, neuropathological and brain uranium concentration analyses, as noted above. All determinations will be made on 3-5 separate samples. The study will be performed in experimental blocks.

The entire project will be conducted at two institutions, Virginia Tech (B. Jortner, principal investigator and neuropathologist and M. Ehrich, neurotoxicologist) and the University of Florida (D. Barber, heavy metal toxicologist), using the following temporal plan.

Months 1-4: Preparations for study (both institutions) and preliminary studies to identify appropriate doses (University of Florida). Consultation on doses (Virginia Tech).

Months 5-12 (kinetic study): Kinetics of DU (as uranyl acetate) in the brain and interaction of stress and DU are studied using 4 treatments (low DU, high DU, low DU + stress, and high DU + stress) at 4 times (e.g. 8 hours, 1 day, 7 days, and 30 days) after a single injection of DU (University of Florida). Data analysis (both institutions).

Months 13-26 (pilot stress study and acute toxicity study): A pilot study of long-term routine stress with periodic superimposed novel stress will be done, using plasma corticosterone levels as a measure. An acute study will be undertaken, assessing toxicity and uranium kinetics of a single intramuscular injection of DU (as uranyl acetate). This will employ 8 treatments (vehicle control, low DU, mid DU, high DU, control + stress, low DU + stress, mid DU + stress and high DU + stress) at 4 times (e.g. 1, 3, 7, and 30 days after injection). Procedures include: in-life study, neurobehavioral assessment, neuropathology (Virginia Tech); tissue uranium assays (University of Florida); neurochemistry, data analysis (both institutions).

Months 27-38 (acute toxicity study, long-term toxicity study): Analysis of samples collected from the acute study continues. In-life portion of long-term (6 month exposure) study is conducted, with assessment of toxicity and uranium kinetics following intramuscular injection of DU pellets. Eight treatments are used, tantalum control, low DU, medium DU, high DU, tantalum + stress, low DU + stress, medium DU + stress, and high DU + stress. Procedures include: in-life study, neurobehavioral assessment, neuropathology (Virginia Tech), uranium assays (University of Florida), neurochemistry, data analysis (both institutions).

Months 39-48 (acute and log-term toxicity studies, sample and data analysis): There is completion of brain uranium tissue assays (University of Florida), in-life portion of chronic study, neuropathology (Virginia Tech), neurochemistry (both institutions) from chronic and acute studies. Assembly of data and preparation of final report is done (both institutions).

Bernard S. Jortner, VMD
Professor of Pathology, Principal Investigator

David Richardson, Director
Virginia Tech Office of Sponsored Programs

Multifactorial Assessment of Depleted Uranium Neurotoxicity (DAMD17-01-1-0775)

Bernard S. Jortner

David Barber

Marion F. Ehrich

Virginia Tech and the University of Florida

Outline of Presentation

- Statement of Problem
- Hypothesis
- Statement of Work Summary
- Analytical Methods
- Aim 1 - DU Kinetics- Methods, Brain Uranium, Nephrotoxicity
- Aim 2a - Acute Toxicity
- Aim 2b - Long-term Toxicity
- Aim 3 - Gene Expression
- Comparative Stress Study
- Summary and Future Tasks

Statement of Problem

- Depleted uranium (DU) is a component of US military weaponry.
- Gulf War experience indicates combat and related activities may lead to exposure to this metal.
- A potential for DU-related neurotoxicity has been demonstrated by studies in humans (McDiarmid) and experimental animals (Pellmar).
- Stress has been noted in some Gulf War deployed troops. Role of stress in possible DU neurotoxicity remains undefined.

Hypotheses

- DU uptake and toxicity in the brain may be region specific and can be altered by stress.
- The DU/stress combination may lead to neurodegeneration.
- The latter can be measured by clinical, neuro-pathological and neurochemical assessments.
- An experimental animal (rat) model is adequate to assess these effects.

Statement of Work

- **Aim** - Characterization of kinetics and toxicity of DU in the nervous system, and the influence of stress, using a male Sprague-Dawley (SD) rat model.
- **Approaches** - Use of a range of DU doses (soluble, particulate), +/- stress, multiple brain regions assessed.
- **Methods of Evaluation** - Determination of tissue uranium concentration, in-life neurobehavioral testing, detailed neuropathology and neurochemistry at sacrifice, data analysis.

Statement of Work (cont.)

- **Specific studies**

  - Specific aim 1: Toxicokinetic (months 1-9) - DU as uranyl acetate, completed.
  - Specific aim 2a: Acute neurotoxicity (months 10-16) - DU as uranyl acetate.
  - Specific aim 2b: Chronic neurotoxicity (months 17-27) - particulate DU.
  - Specific aim 3: Follow-up (gene expression) study (months 28-36).

Analytical Methods

- **Uranium kinetics** - inductively coupled plasma-mass spectroscopy (ICP-MS).
- **Neurobehavior** - Functional Observational Battery (FOB), motor activity, rotorod performance, passive-avoidance.
- **Neuropathology** - perfusion-fixation, multilevel sampling of nervous system.

  Paraffin-embedding - routine stains, immunohistochemistry (glial fibrillary acid proteins, other), Fluoro-Jade® neurodegeneration stain.

  Epoxy resin-embedding - spinal cord and peripheral nerves, 1µm thick sections.

Analytical Methods (cont.)

- **Neurochemistry** (cortex, hippocampus, striatum, cerebellum)

Neurotransmitters	Methods
dopamine, norepinephrine, serotonin	reversed phase high pressure liquid chromatography (HPLC) with electrochemical detection
acetylcholine	gas chromatography - mass spectroscopy
glutamate, GABA	HPLC with fluorometric determination

Analytical Methods (cont.)

Neurotransmitter receptor quantitation	Methods
serotonin, acetylcholine (nicotinic and muscarinic), dopamine, GABA	saturation radioligand binding

Oxidative stress	Methods
reduced and oxidized glutathione	HPLC with fluorometric detection
glutathione peroxidase, superoxide dismutase	Oxyscan antioxidant analyzer

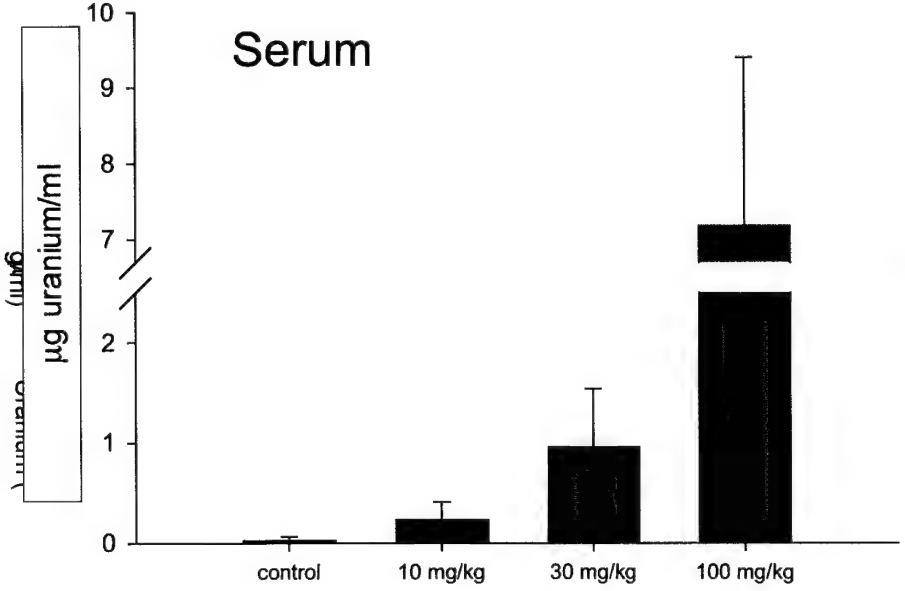
Analytical Methods (cont.)

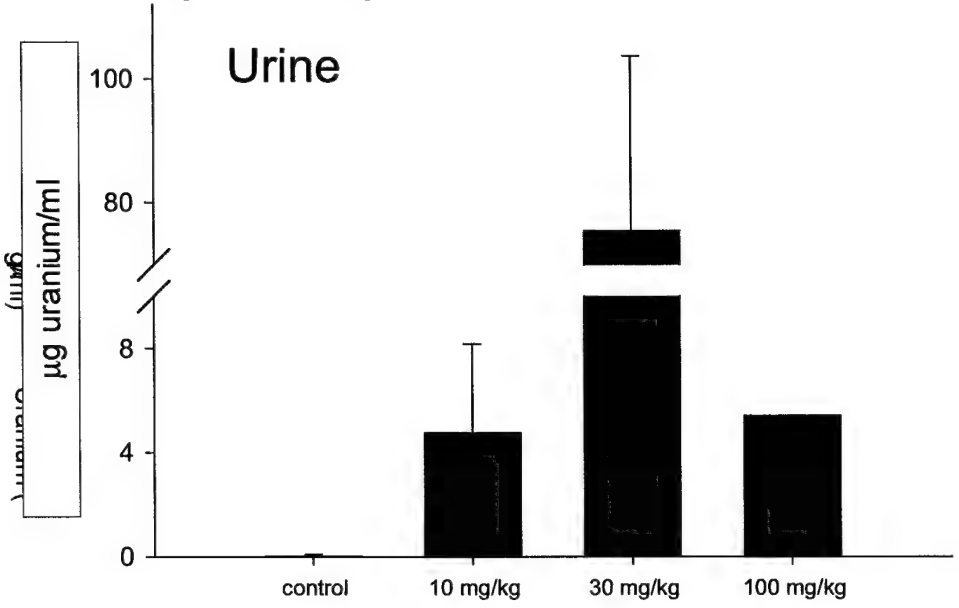
- **Regional Gene Expression in Brain** - rat neurobiology microarray
- Affymetrix U34A Rat Neurobiology Array.

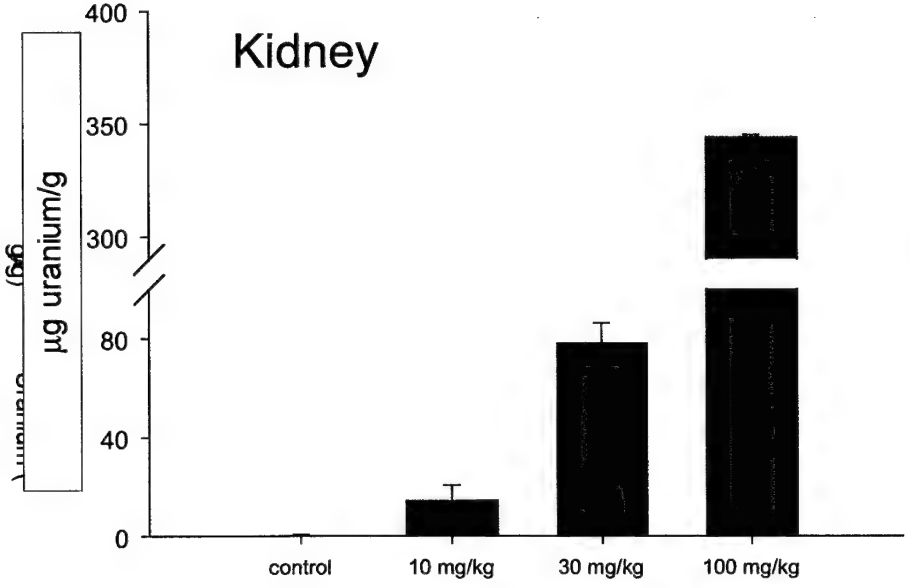
Aim 1: Preliminary studies

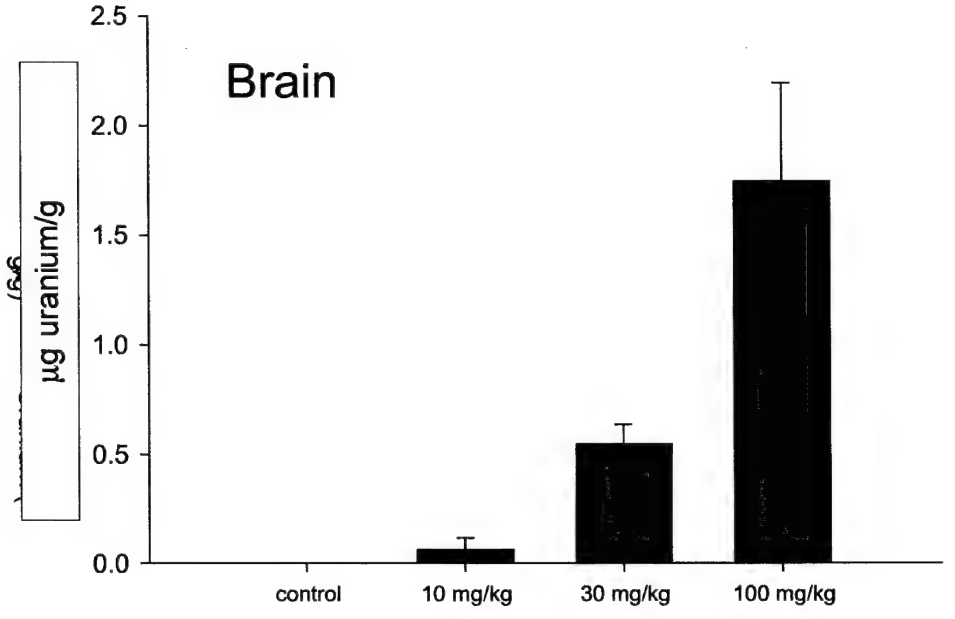
- Male SD rats given uranyl acetate in saline intraperitoneally (IP) at 10, 30, or 100 mg U/kg (n=3).
- Animals housed in metabolism cages.
- 24 hours after dosing, serum, brain, kidney, and urine (24 hour) samples obtained.
- Uranium concentrations determined by ICP-MS.
- Doses chosen - 1, 10 mg U/kg.

Aim 1: Preliminary Study









Aim 1: Kinetics of soluble DU in brain

- Forced swim stress applied for 5 days prior to DU (1 or 10 mg U/kg) exposure.
- Samples obtained 8 hours, 24 hours, 7 days, 10 days (10mg/kg only), or 30 days (1mg/kg only) after exposure.
- Uranium determined by ICP-MS following tissue digestion.

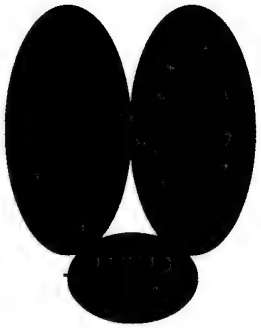
Aim 1: Experimental Design

- Male SD rats (~250 g) were randomly assigned to stress or control groups (n=40/group).
- Stress animals were subjected to 12-minute periods of forced swimming for 5 days prior to DU exposure; controls handled similarly but no swimming.
- DU injections given 1 hour after last episode of swimming.

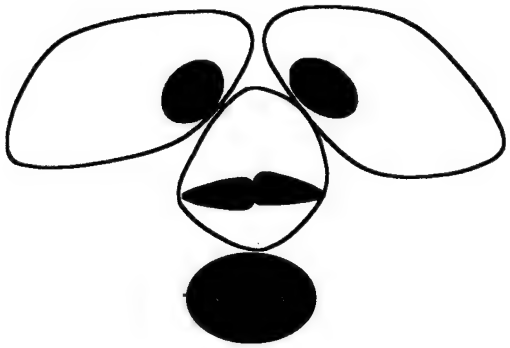
Aim 1: Design cont.

- Animals given IP injections of uranyl acetate in saline at 1 or 10 mg U/kg.
- Animals housed under normal conditions after DU exposure.
- At 8 hours, 24 hours, 7 days, 10 days or 30 days (n=5/group), animals were killed by CO_2. Serum, kidney, liver, and brain samples were collected.

Brain Dissection



Intact Brain



Dissected Brain

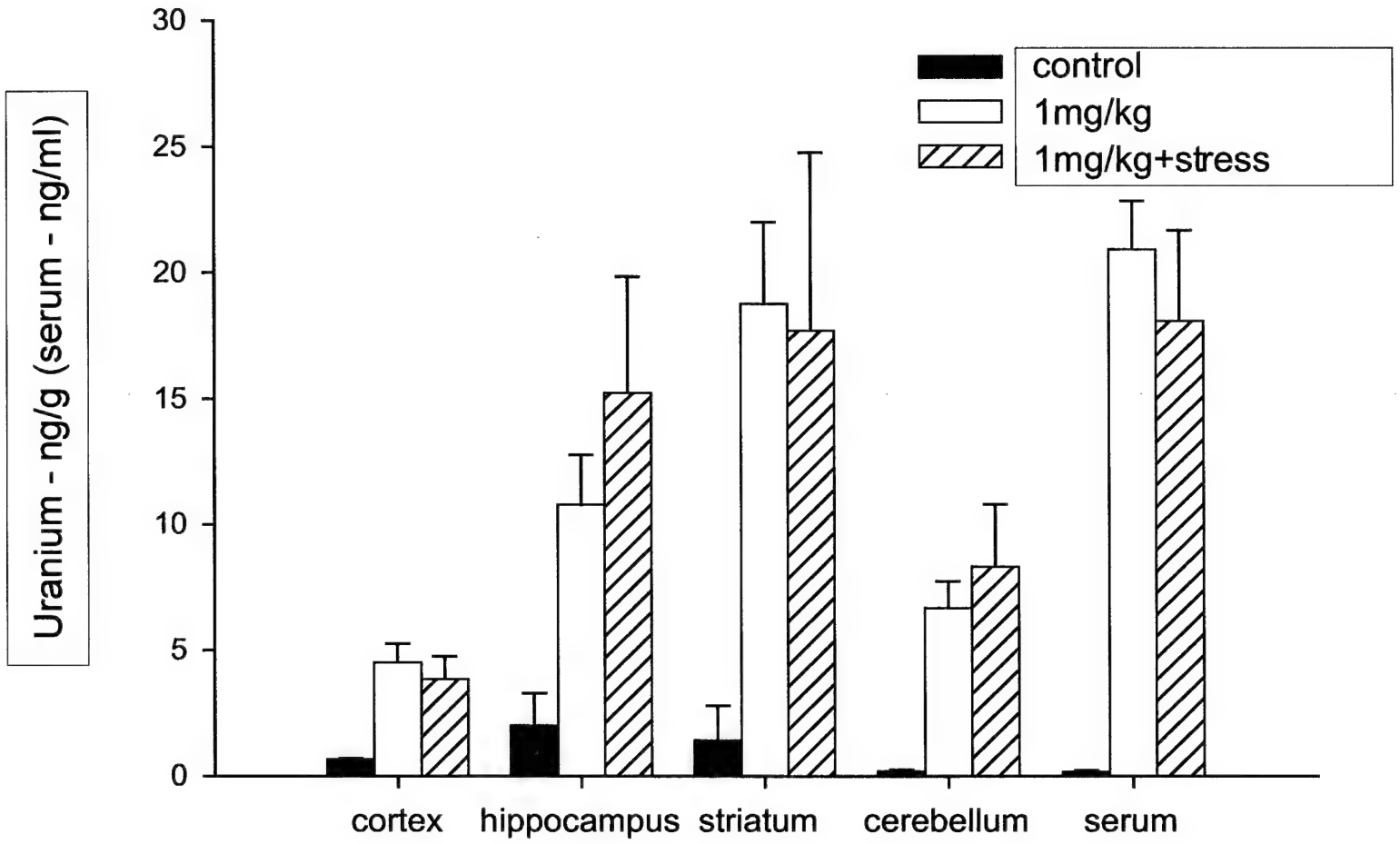
Aim 1: Sample preparation for ICP-MS analysis

- Digestion with nitric acid and hydrogen peroxide in glass pressure tubes at 140°C.
- Dilution to 5 ml, 0.45 micron filtration.
- Recovery from spiked samples was determined to be 96-108%.

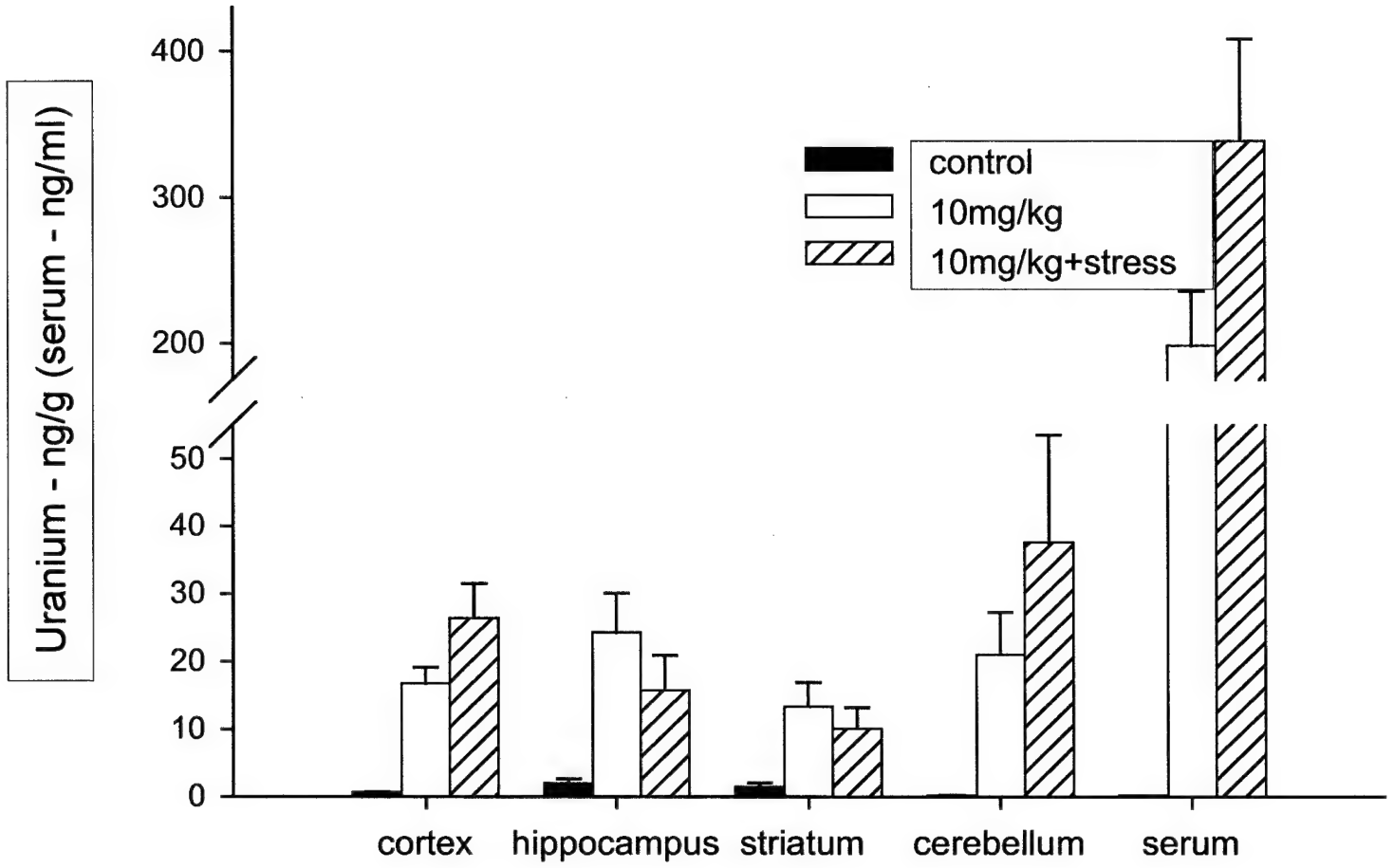
Uranium Analysis

- Inductively coupled plasma-mass spectrometry (ICP-MS)- HP 7500a (Analytical Section, Hazard Identification Core, University of Arizona).
- Uranium determined from m/z 238.
- Iridium used as internal standard.
- 5 repetitions performed/sample to obtain value.
- Limit of detection- 2 ppt.

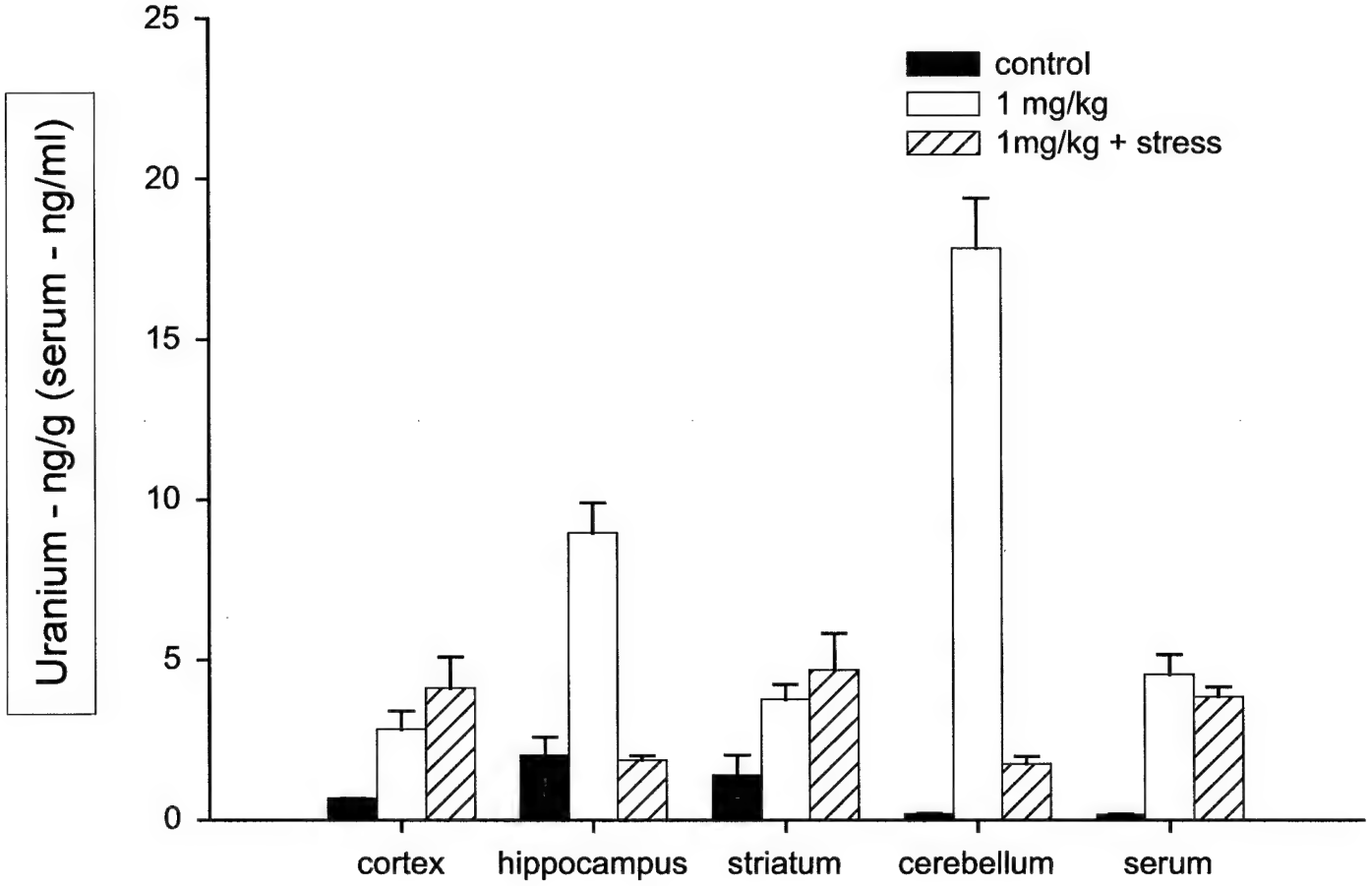
Aim 1: Brain Uranium 8 hours after 1 mg/kg



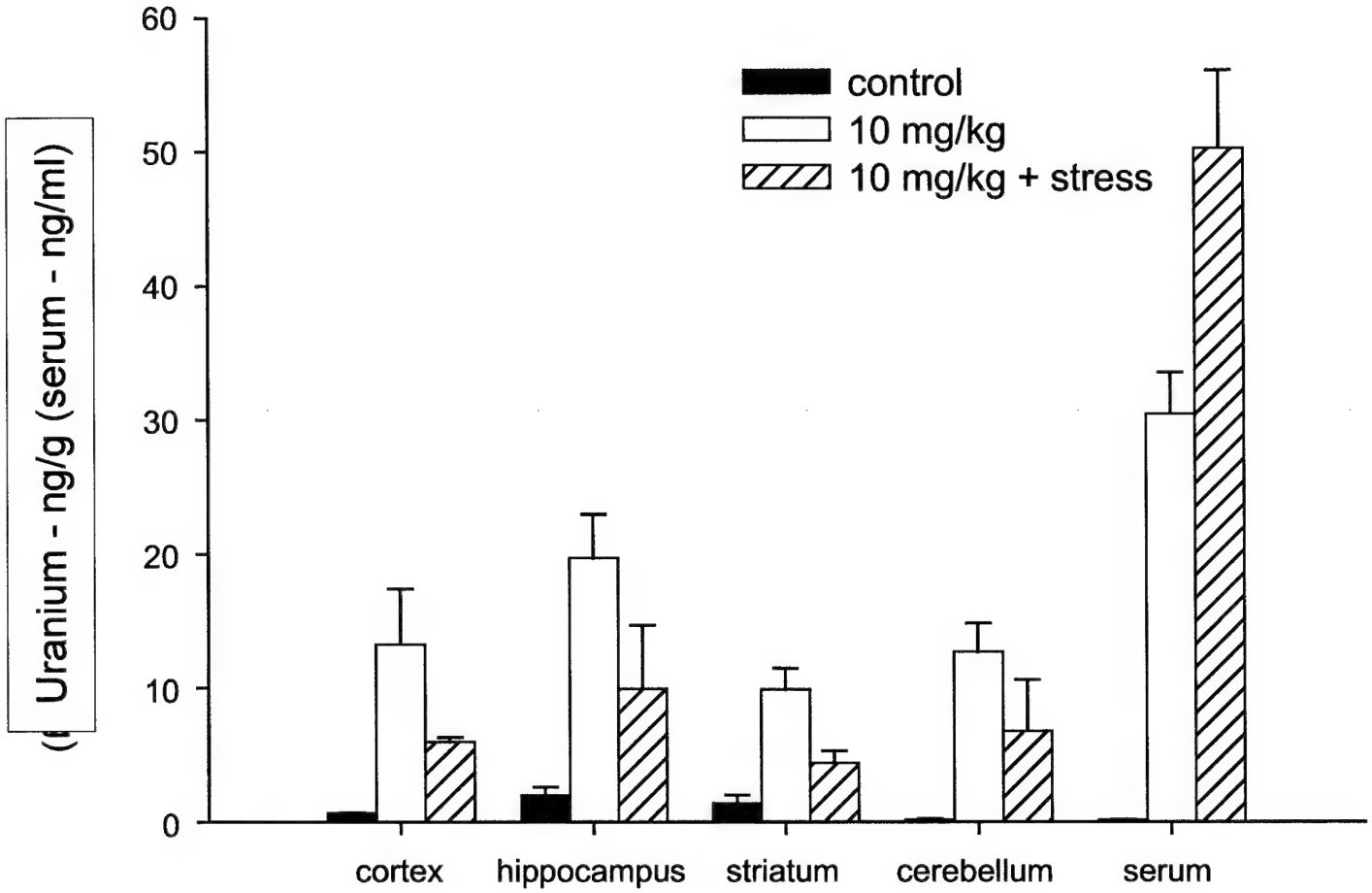
Aim 1: Brain Uranium
8 hours after 10 mg/kg



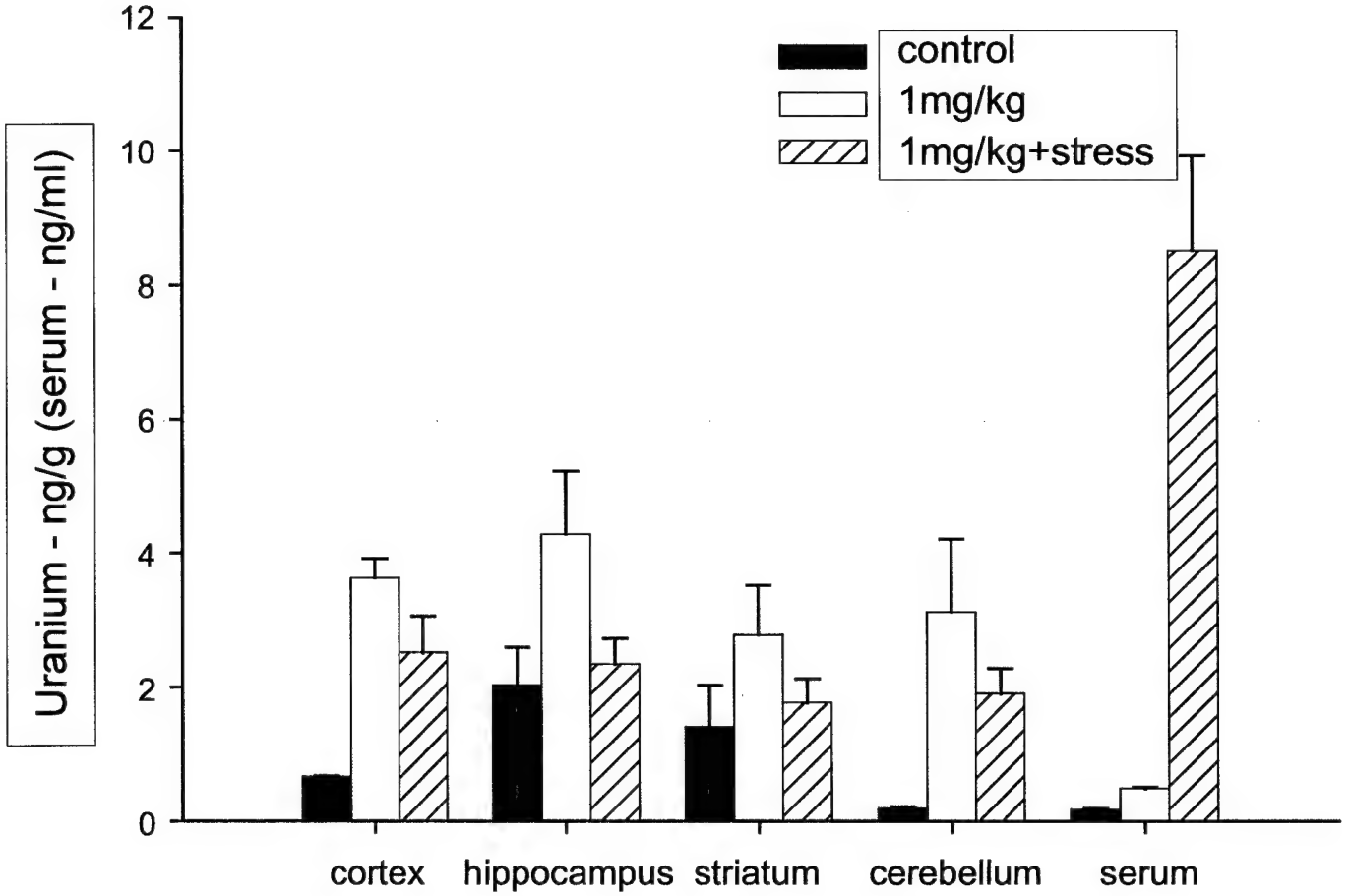
Aim 1: Brain Uranium 24 hours after 1mg/kg



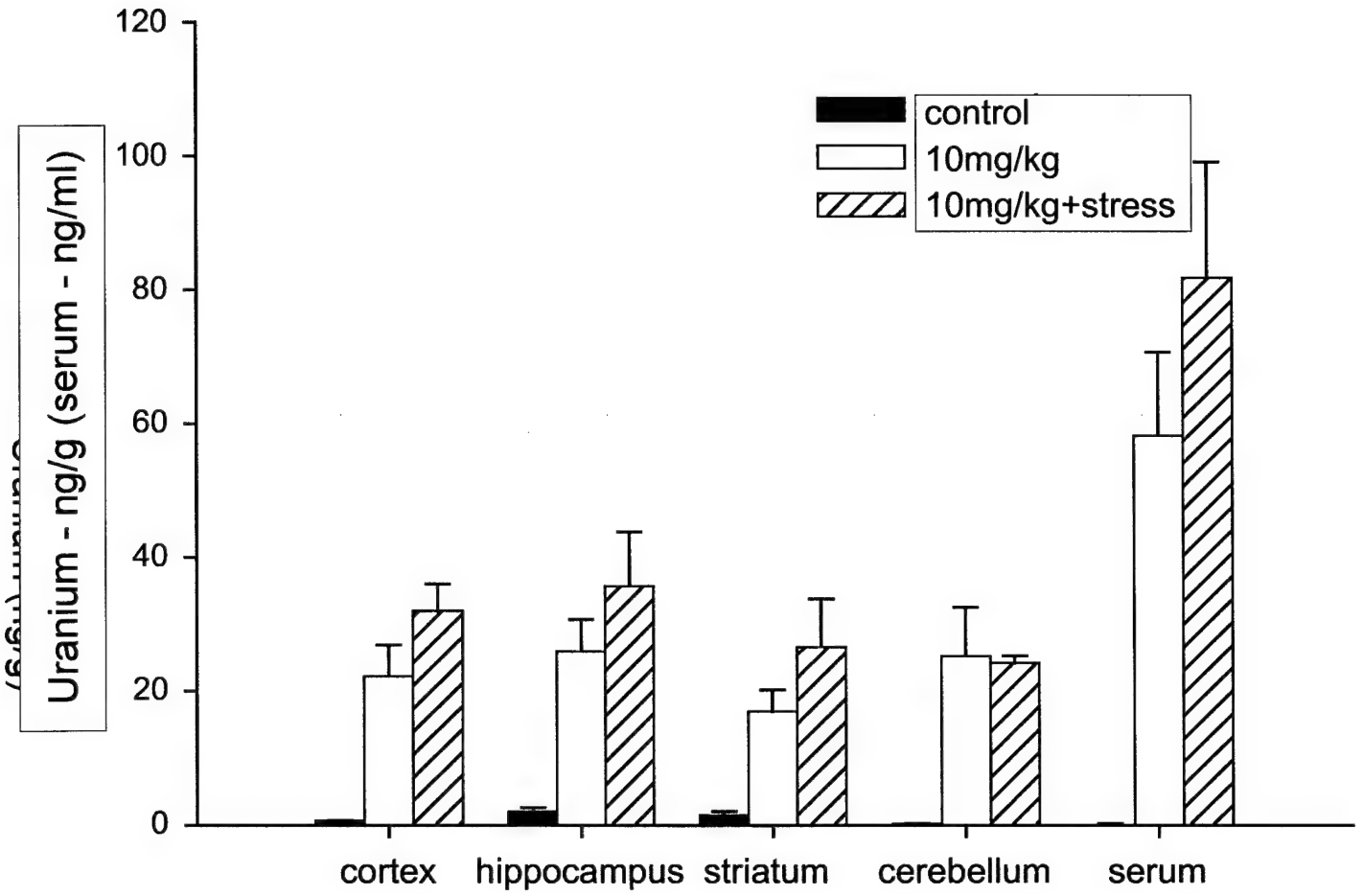
Aim 1: Brain Uranium 24 hours after 10 mg/kg



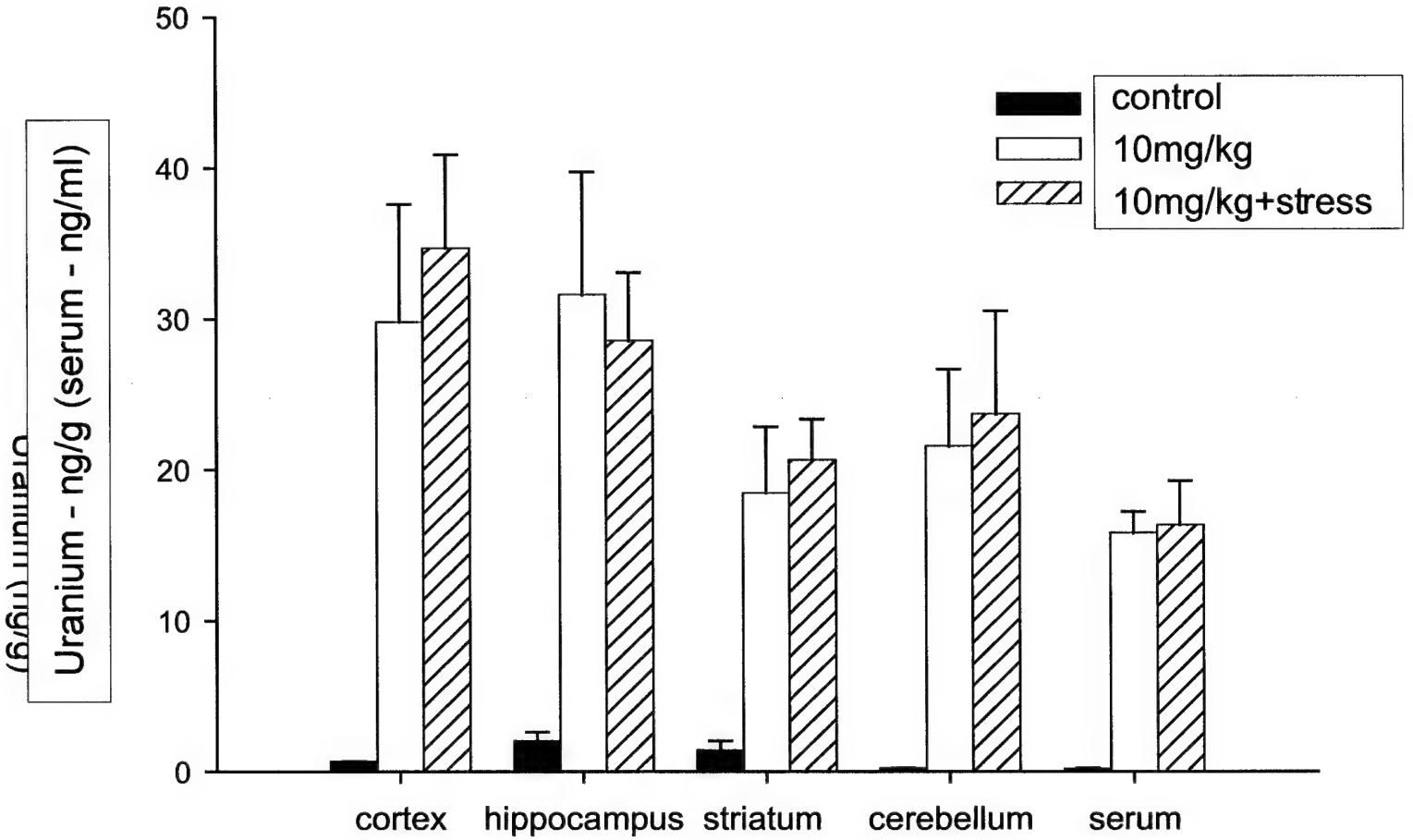
Aim 1: Brain uranium 7 days after 1mg/kg



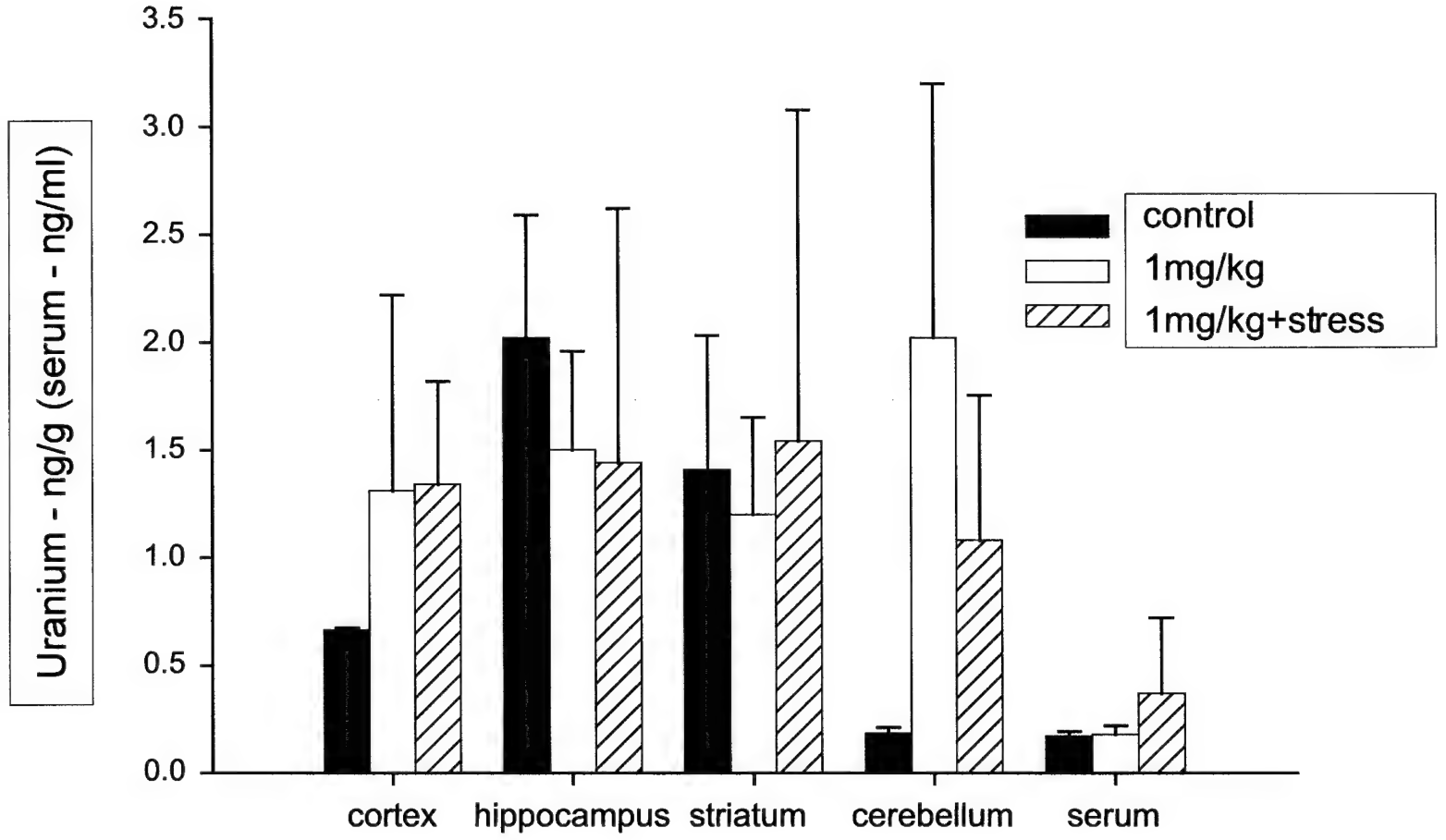
Aim 1: Brain Uranium
7 days after 10 mg/kg



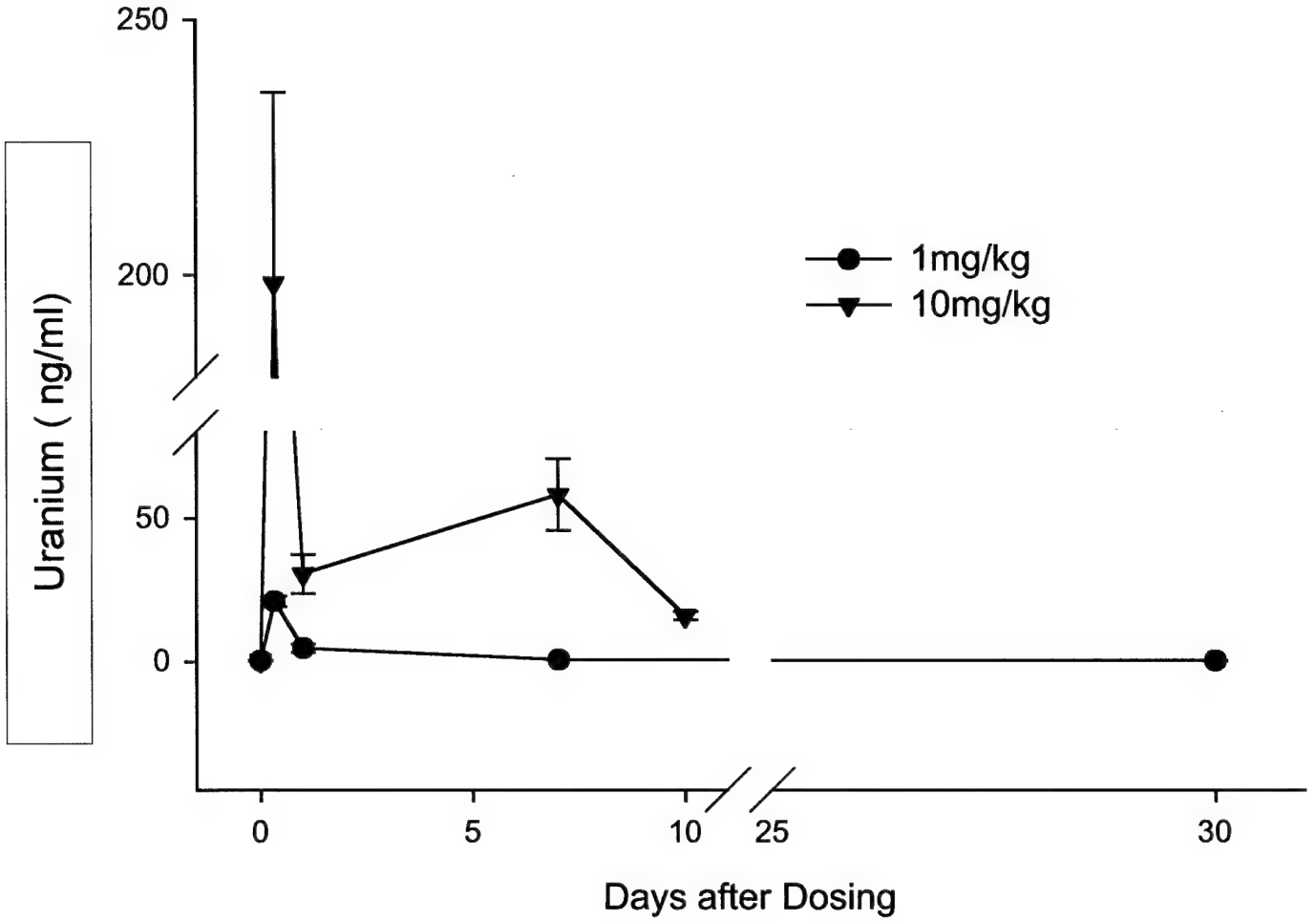
Aim 1: Brain Uranium 10 days after 10 mg/kg



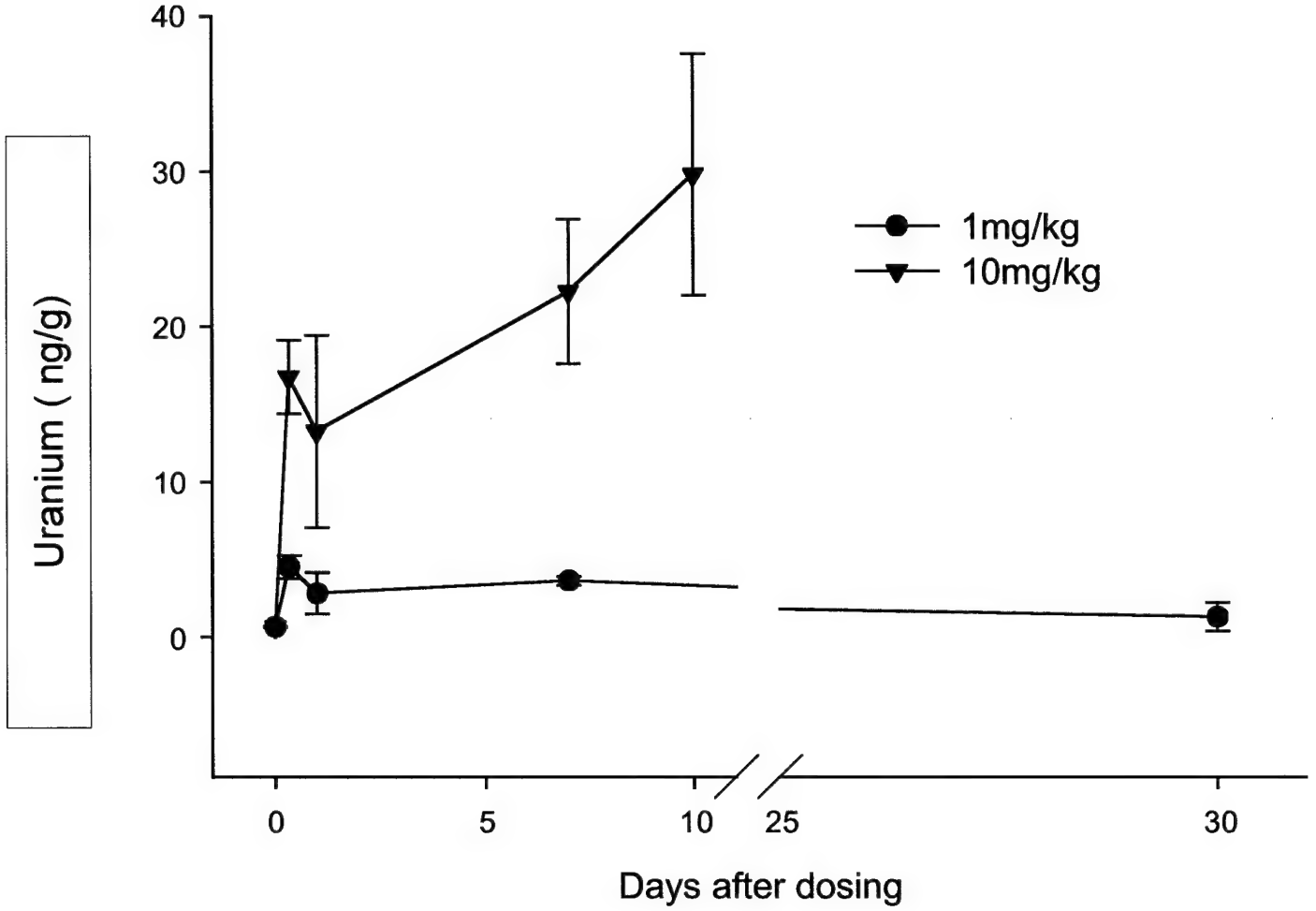
Aim 1: Brain Uranium 30 days after 1mg/kg



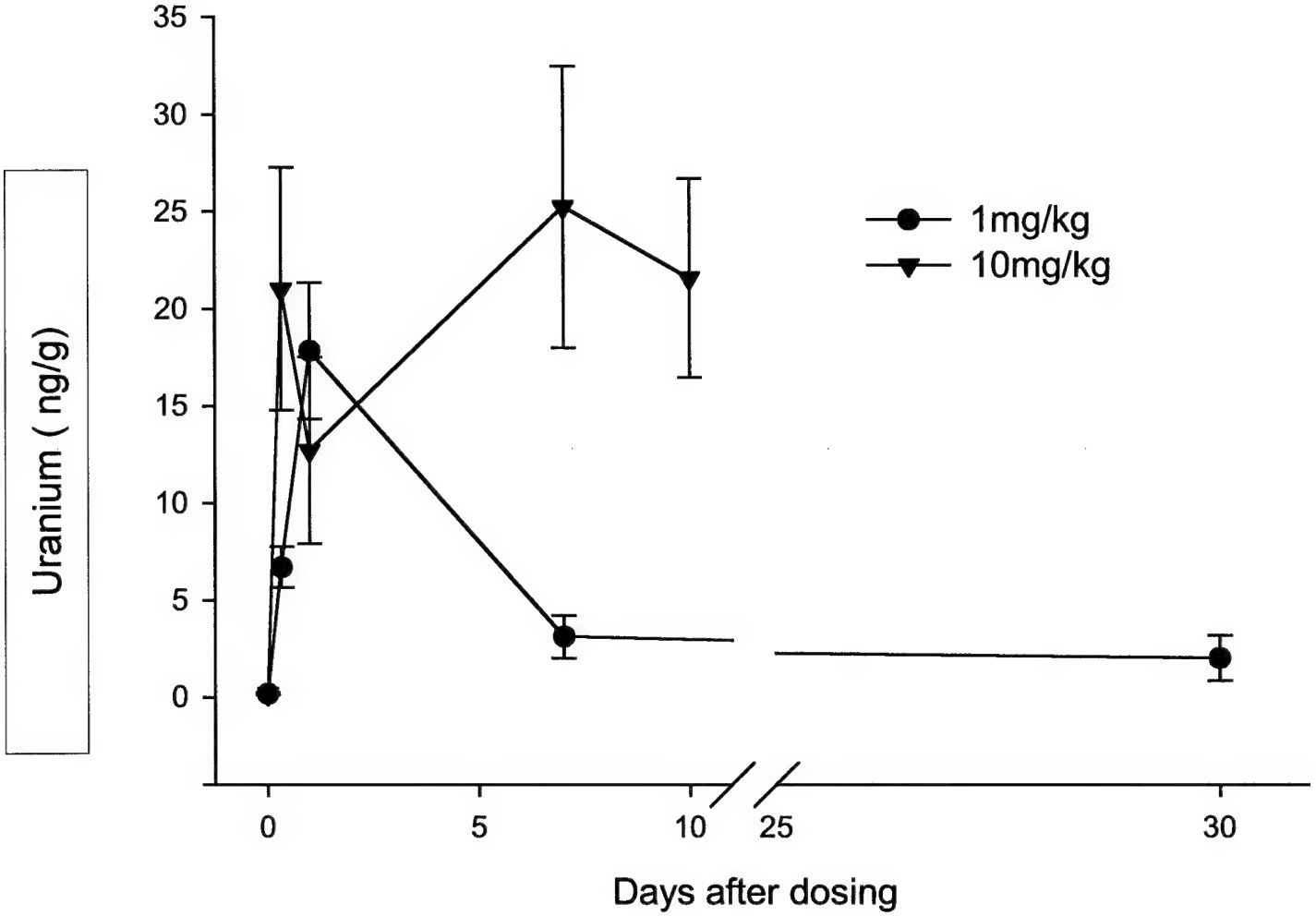
Aim 1: Uranium in serum



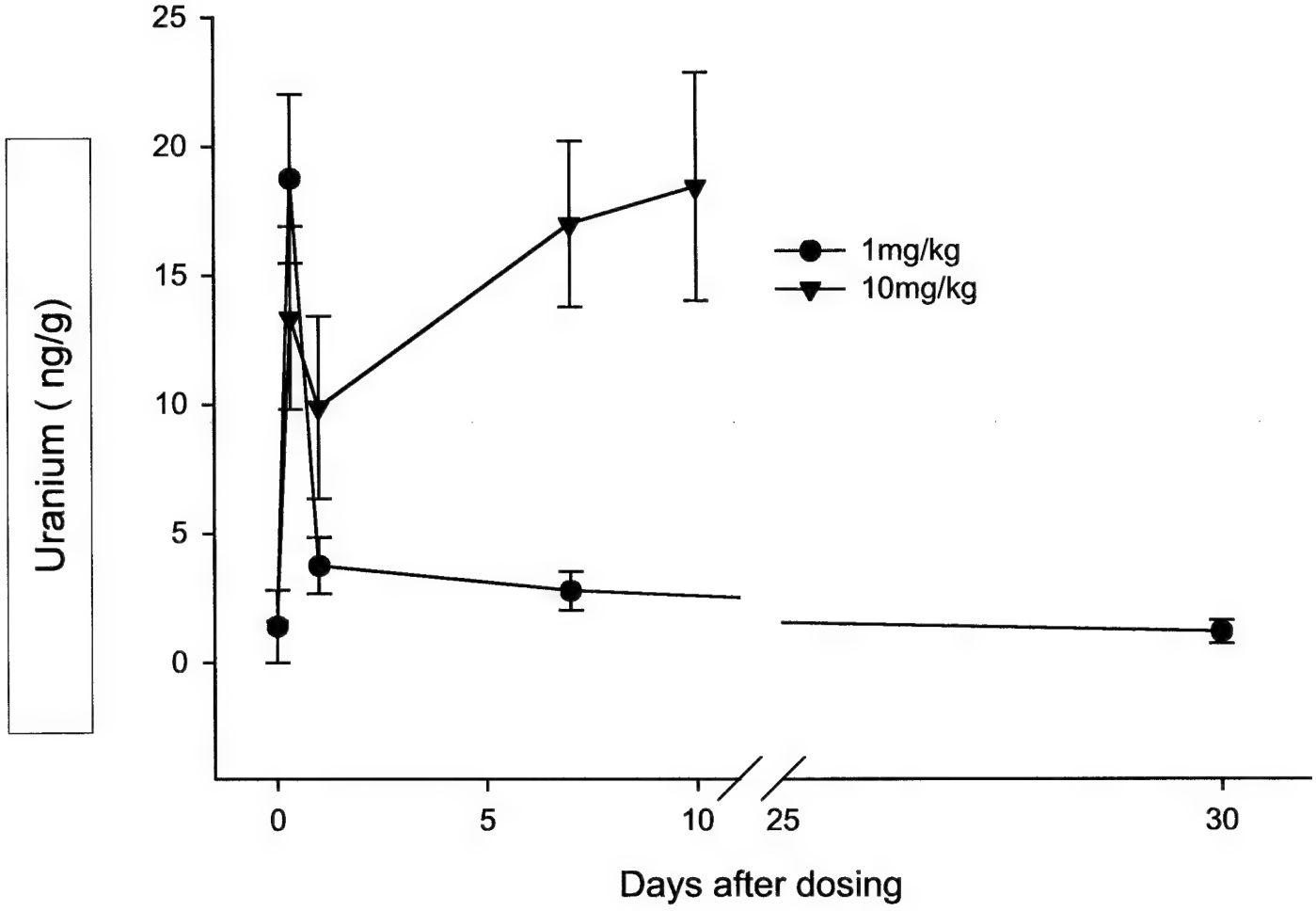
Aim 1: Uranium in cortex



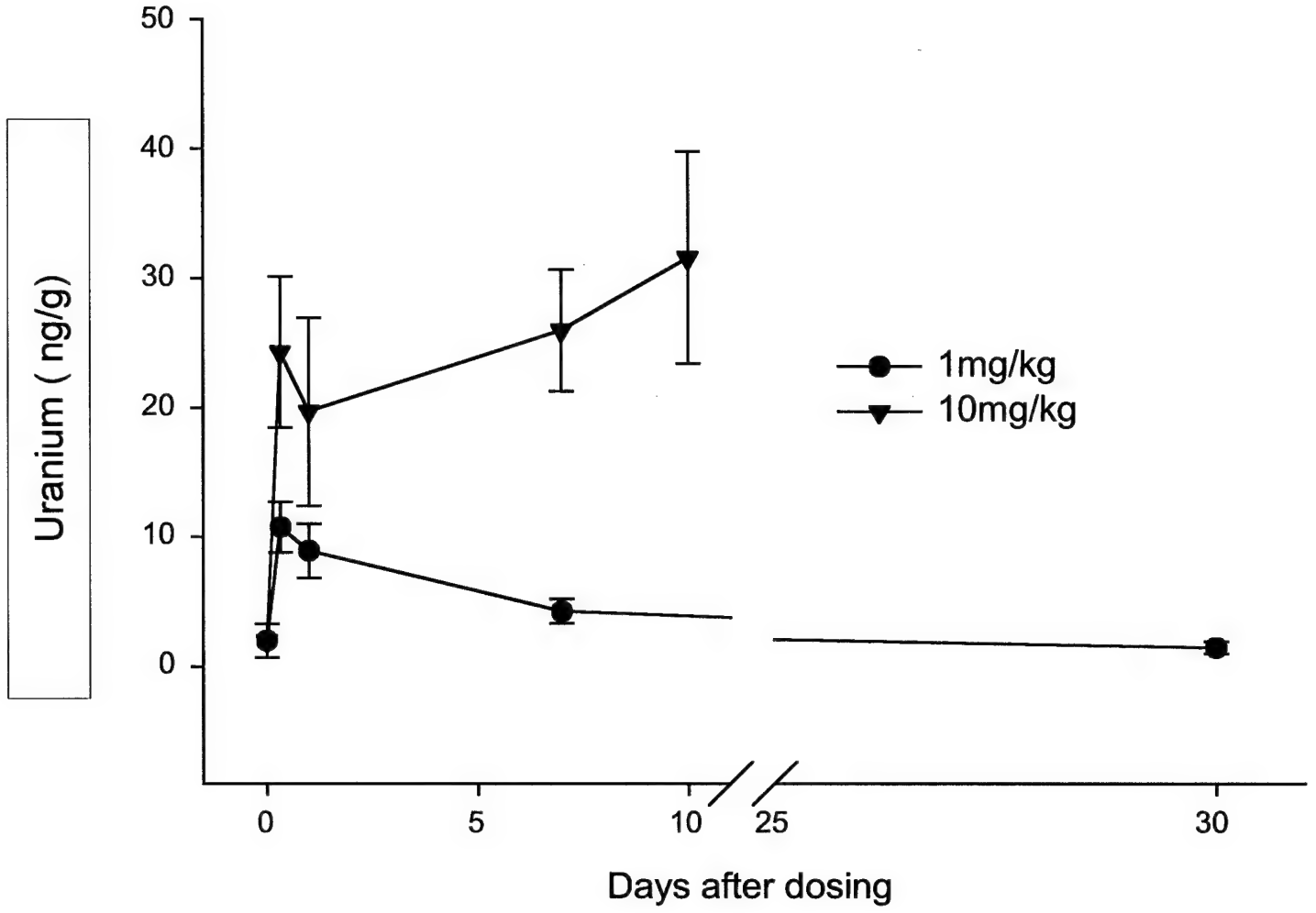
Aim 1: Uranium in cerebellum



Aim 1: Uranium in striatum



Aim 1: Uranium in hippocampus



Aim 1: Conclusions

- A single exposure to 1mg soluble U/kg increased regional brain uranium levels for at least 7 days; especially in cerebellum
- A single 10mg/kg dose increased uranium levels in all brain regions examined, exhibiting a delayed peak
- Brain levels observed were similar to those reported by Pellmar et al.
- No apparent effect of stress on brain uranium

Depleted Uranium Nephrotoxicity (Supplement to Aim 1)

- **Design** (tissue from kinetic study - Specific Aim 1)
  - Male Sprague-Dawley rats
  - Dosages: 0, 1 or 10 mg/kg DU (as uranyl acetate intraperitoneally)
  - Swim stress/no stress, for 5 days prior to dosing
  - Sacrifice: 8, 24, 168, 240 (10 mg/kg only), 720 (1 mg/kg only) hours
  - n = 3-5/group

Depleted Uranium Nephrotoxicity (cont.)

Clinical Data

- Serum Uranium Concentration (ng/ml)

Hours	DU dose (mg/kg)	
	1	10
8	20.9+/-1.9	198.4+/-37.3
24	4.5+/-0.6	30.5+/-3.1

n = 3-5/group

- Mortality - 3/10 10 mg/kg rats died - day 9.

Depleted Uranium Nephrotoxicity (cont.)

- **Renal Pathology**

Proximal Tubular Necrosis - 1 mg/kg

Hours	Stress	No Stress
8	not remarkable (5/5)	not remarkable (5/5)
24	minimal, focal (2/5)	minimal, focal (1/3)
168	mild-moderate + regeneration (4/5)	mild-moderate + regeneration (3/5)
720	regeneration (3/5)	regeneration (3/5)

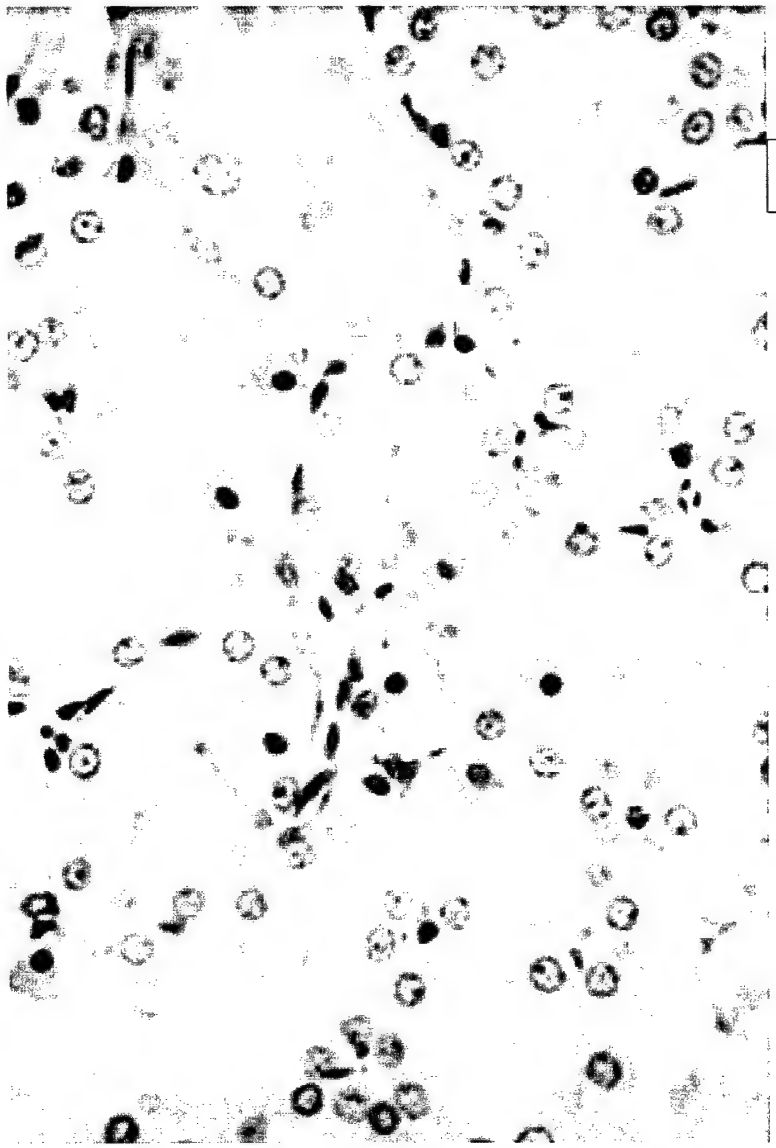
Depleted Uranium Nephrotoxicity (cont.)

- **Renal Pathology (cont.)**

Proximal Tubular Necrosis - 10 mg/kg

Hours	Stress	No Stress
8	minimal - mild, focal (4/5)	minimal - mild, focal (2/5)
24	moderate (3/5)	mild - moderate (3/5)
168	marked + regeneration (5/5)	marked + regeneration (5/5)
720	marked + regeneration (3/3)	marked + regeneration (4/4)

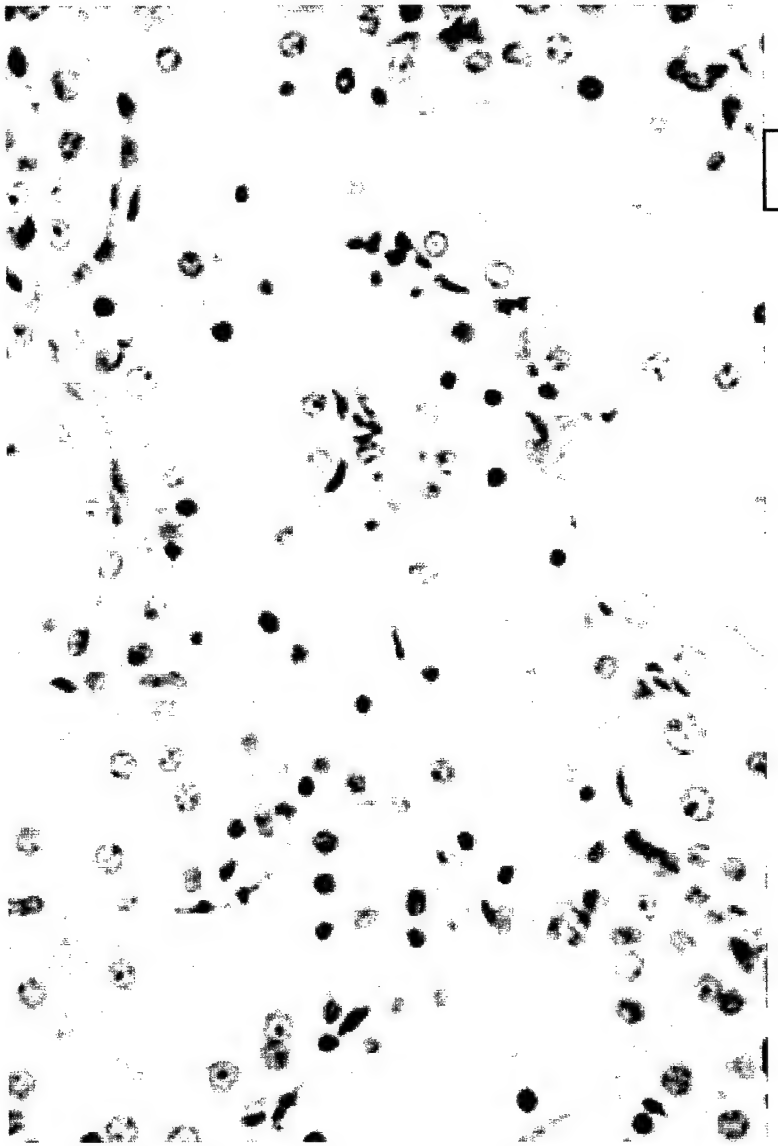
DU Toxicity Kidney



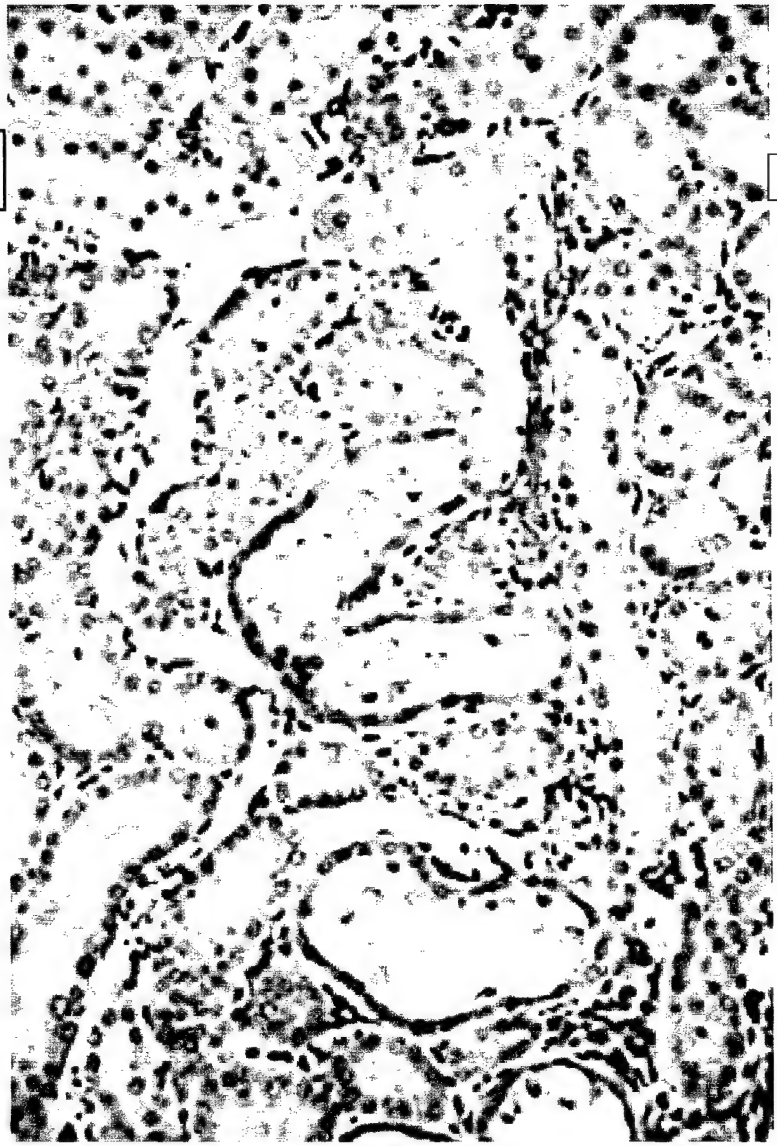
1 mg/kg, 24 hours, H&E

10 mg/kg, 24 hours, TUNEL

DU Toxicity Kidney



64 10 mg/kg, 24 hours, H&E



1 mg/kg, 168 hours, H&E

Depleted Uranium Nephrotoxicity (cont.)

- **Summary**
  - Soluble DU is nephrotoxic (Sanchez *et al.*; Lim *et al.*).
  - Lesions include tubular epithelial necrosis, with some apoptosis (TUNEL +).
  - A dose-effect is seen at 1 and 10 mg/kg; mortality with 10 mg/kg.
  - Tubular regeneration is prominent in later stages.

Aim 2a: Acute Toxicity

- Behavior (FOB, motor activity, passive avoidance), pathology and neurochemical determinations; +/- stress (forced swimming daily $\geq$ 5 days before dose); single intraperitoneal dose of uranyl acetate [high/low] determined by Aim 1; 1 day, 7 day and 30 day sacrifices.

sacrifice	vehicle	low UA	high UA
1 day (no pathology)	+stress	+stress	+stress
7 days			
30 days			
1 day (no pathology)	-stress	-stress	-stress
7 days			
30 days			

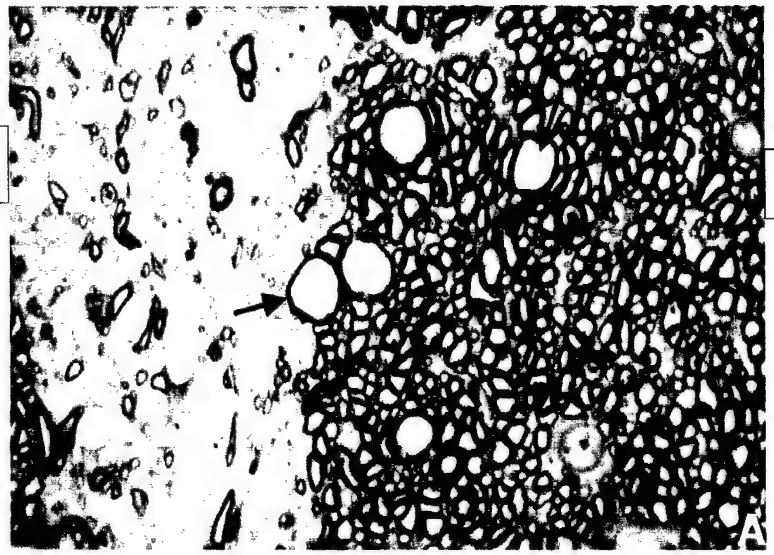
n=6 groups, 3-4 animals/group for pathology (except day 1 sacrifice), $\geq$ 5 animals/group for neurochemical study at each time point.

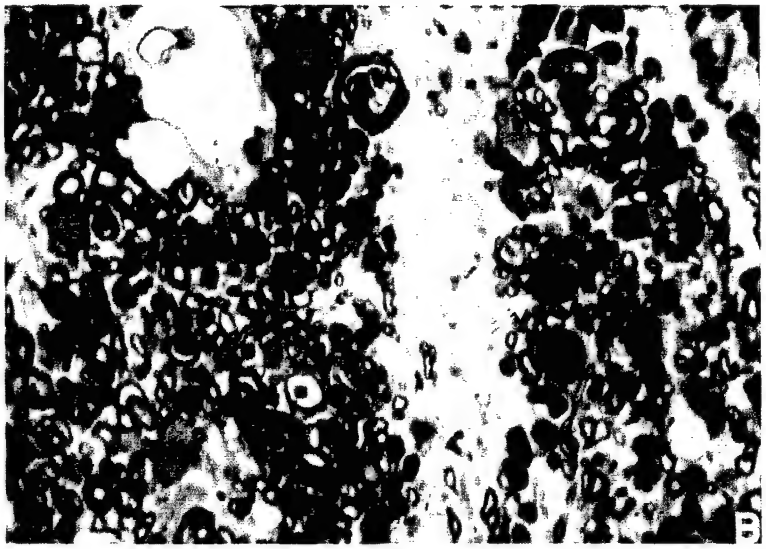
Plasma corticosterone will be measured on the day of DU administration, 1 hour after cessation of stress treatment.

Functional Observational Battery (FOB)
Observations and Measurements

Home Cage	Test Name
	posture
	clonic involuntary movement
	tonic involuntary movement
	vocalizations
	respirations
	activity level
Handling	
	cage removal ease
	lacrimation
	salivation
	piloerection
	palpebral closure
	palpebral reflex
	pupil size
	pupil light response
	oculocardiac
	visual placing
	handling reactivity

Open field	Test Name
	clonic involuntary movement
	tonic involuntary movement
	gait
	gait score
	mobility score
	arousal
	rearing
	fecal boluses
	urine pools
	stereotypy
	bizarre behavior
Reflexes	
	approach response
	touch response
	click response
	tail pinch response
	righting reflex
	tail-limb
Physiological	
	body weight
	temperature
	rotorod
	forelimb grip
	hindlimb grip
	landing foot splay
	other





GFAP

Toluidine blue - Safranin

Aim 2b: Long-term Toxicity

- Neurobehavioral observations (before dosing and every three weeks); uniformly sized DU particles injected into quadriceps; +/-stress (daily for 14 days after DU injection); pathology, neurochemical determinations at 6 month terminal sacrifice.

tantalum control	low UA	medium UA	high UA
+stress	+stress	+stress	+stress
-stress	-stress	-stress	-stress

n=8 groups, 3-4 animals/group for pathology, ≥5/group for neurochemistry.

Plasma corticosterone will be measured on the day of DU administration 1 hour after cessation of stress treatment.

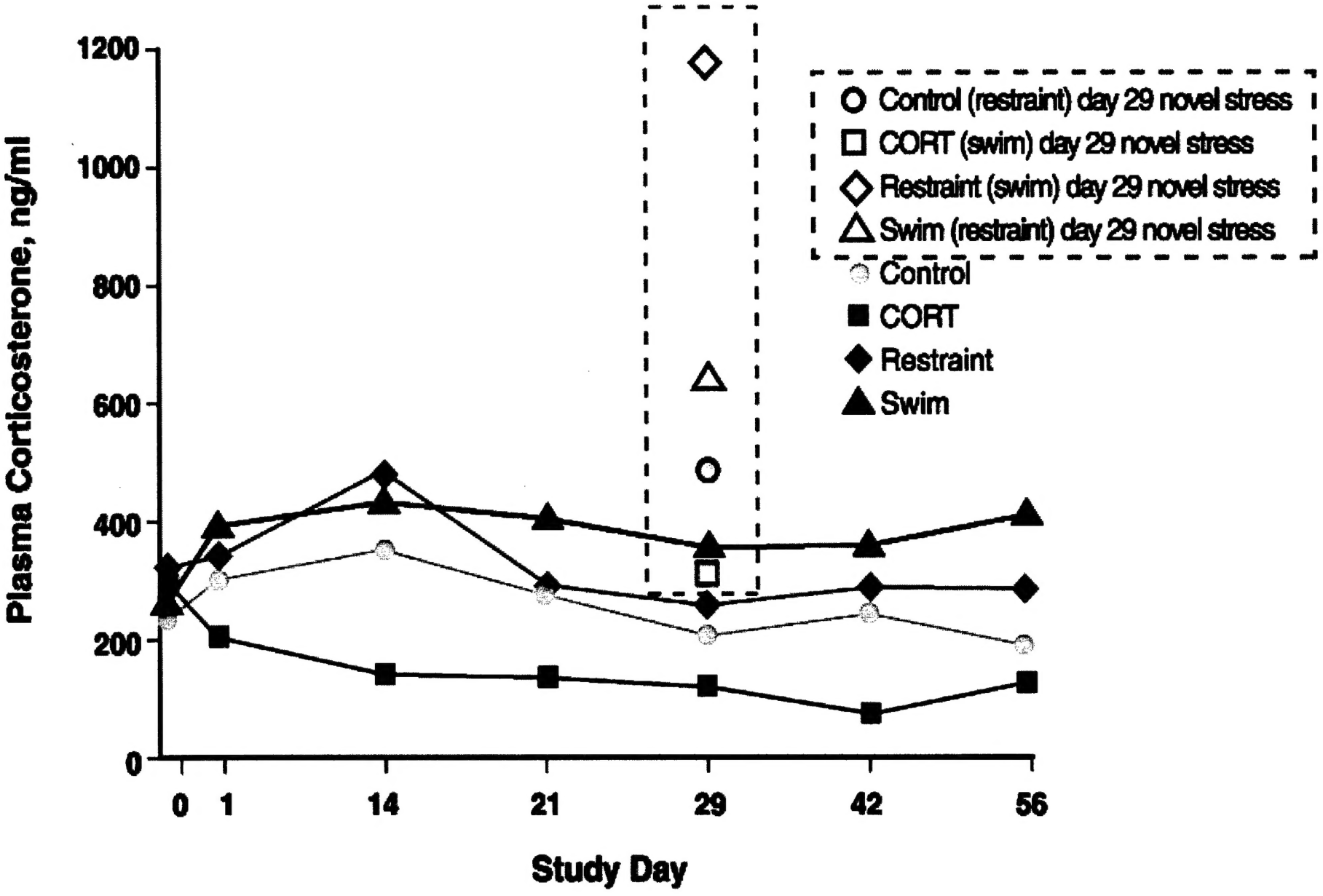
Aim 3: Gene Expression

- **Gene expression** - 3 groups (control, DU, DU+stress), 1 dose, 1 time point, brain region(s) determined from Aims 1 and 2.
- 3 animals/group.
- Affymetrix U34A Rat Neurobiology Array.
- UF ICBR Microarray Core.

Comparative Stress Study

- Stressors - routine handling (control)
  restraint
  swim
  } 5 days/week
  corticosterone (400 μg/ml) in drinking water
- n = 10/group (5 sacrificed on day 29 following novel stress,
  5 on day 62)
- Measures - plasma corticosterone
  clinical biochemistry
  organ/body weights
  histopathology (adrenal, brain, spleen, thymus)

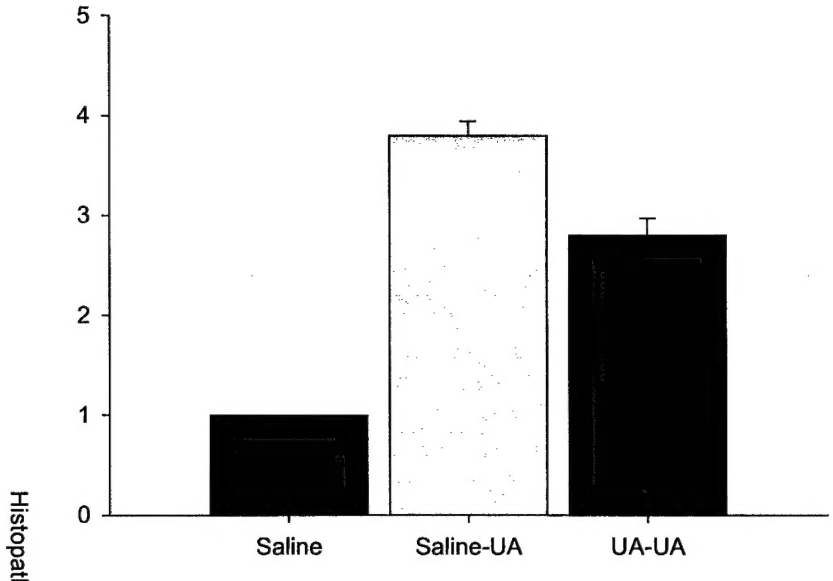
Comparative Stress Study

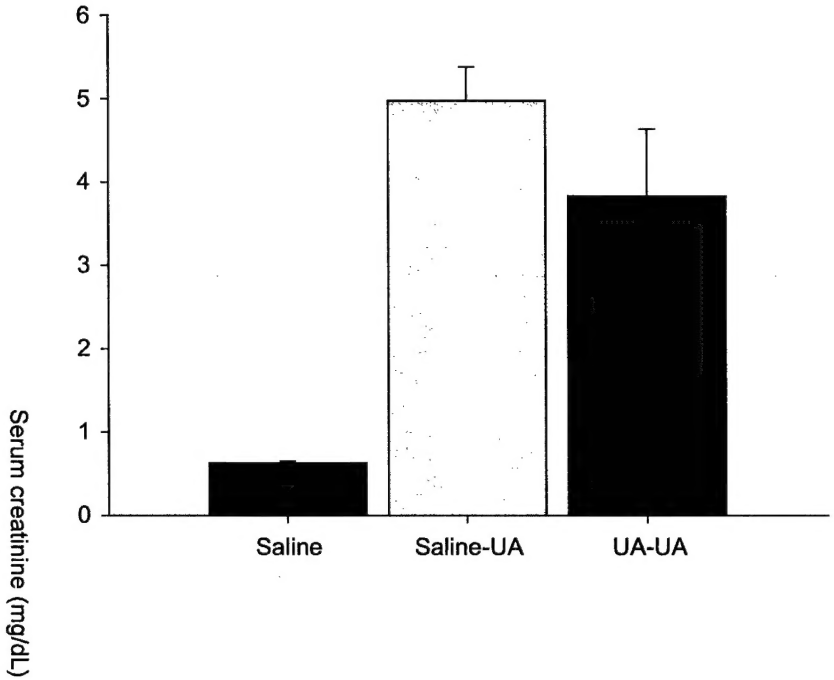


Summary and Future Tasks

- **Completed**
  - Regional kinetics of soluble DU in brain (specific aim 1)
  - Renal toxicity of soluble DU (specific aim 1)
  - Comparative stress study
- **To be done**
  - Acute neurotoxicity (specific aim 2a)
  - Long-term neurotoxicity (specific aim 2b)
  - Gene expression (specific aim 3)
  - Overall interpretation of study

Reduced nephrotoxicity after uranium challenge





Hsp induction and uranium nephrotoxicity